(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 October 2001 (25.10.2001)

PCT

(10) International Publication Number
WO 01/79520 A1

(51) International Patent Classification[7]: C12P 19/00, 19/62, C07H 17/08, C12N 1/00, 1/21, 15/54, 15/76

(21) International Application Number: PCT/GB01/01743

(22) International Filing Date: 17 April 2001 (17.04.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0009207.2 13 April 2000 (13.04.2000) GB
09/694,218 23 October 2000 (23.10.2000) US

(71) Applicant *(for all designated States except US)*: **BIOTICA TECHNOLOGY LIMITED** [GB/GB]; 112 Hills Road, Cambridge, Cambridgeshire CB2 1PH (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LEADLAY, Peter, Francis** [GB/GB]; 17 Clarendon Road, Cambridge, Cambridgeshire CB2 2BH (GB). **STAUNTON, James** [GB/GB]; 29 Porson Road, Cambridge, Cambridgeshire CB2 2ET (GB). **GAISSER, Sabine** [DE/GB]; 37 Gwydir Street, Cambridge, Cambridgeshire CB11 2LG (GB).

(74) Agents: **KIDDLE, Simon, J.** et al.; Mewburn Ellis, York House, 23 Kingsway, London, Greater London WC2B 6HP (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/79520 A1

(54) **Title:** HYBRID GLYCOSYLATED PRODUCTS AND THEIR PRODUCTION AND USE

(57) **Abstract:** The present invention relates to hybrid glycosylated products, and in particular, to natural products such as polyketides and glycopeptides, and to processes for their preparation. The invention is particularly concerned with recombinant cells in which a cloned microbial glycosyltransferase can be conveniently screened for its ability to generate specific glycosylated derivatives when supplied with polyketide, peptide, or polyketide-peptides as substrates. The invention demonstrates that cloned glycosyltransferases when rapidly screened for their ability to attach a range of activated sugars to a range of exogenously supplied or endogenously generated aglycone templates, show a surprising flexibility towards both aglycone and sugar substrates, and that this process allows the production of glycosylated polyketides in good yield. This overcomes the problem not only of supplying novel sugar attachments to individual polyketides, including polyketides altered by genetic engineering, but also of increasing the diversity of polyketide libraries by combinatorial attachment of sugars.

Hybrid Glycosylated Products and Their Production and Use

Field of the Invention

The present invention relates to hybrid glycosylated products, and in particular, to natural products such as polyketides and glycopeptides, and to processes for their preparation. The invention is particularly concerned with recombinant cells in which a cloned microbial glycosyltransferase can be conveniently tested for its ability to generate specific glycosylated derivatives when supplied with polyketide, peptide, or polyketide-peptides as substrates.

Background to the Invention

Glycosylation is important for the bioactivity of many natural products, including antibacterial compounds such as the polyketide erythromycin A and the glycopeptide vancomycin, and antitumour compounds such as the aromatic polyketide daunorubicin and the glycopeptide-polyketide bleomycin. Polyketides are a large and structurally diverse class of natural products that includes many compounds possessing antibiotic or other pharmacological properties, such as erythromycin, tetracyclines, rapamycin, avermectin, monensin, epothilones and FK506. In particular, polyketides are abundantly produced by *Streptomyces* and related actinomycete bacteria. They are synthesised by the repeated stepwise condensation of acylthioesters in a manner analogous to that of fatty acid biosynthesis. The greater structural diversity found among natural polyketides arises from the selection of (usually) acetate or propionate as "starter" or "extender" units; and from the differing degree of processing of the β-keto group observed after each condensation. Examples of processing steps include reduction to β-hydroxyacyl-, reduction followed by dehydration to 2-enoyl-, and complete reduction to the saturated acylthioester. The stereochemical outcome of these processing steps is also specified for each cycle of chain extension. The polyketide chains are usually cyclised in specific ways and subject to further enzyme-catalysed modifications to produce the final polyketide. Naturally-occurring peptides produced by non-ribosomal peptide synthetases are likewise synthesised by repeated stepwise assembly, in this case of activated amino

acids, and the chains produced are similarly subject to further modifications to produce the fully bioactive molecules. Mixed polyketide-peptide compounds, hereinafter defined as incorporating both ketide and amino acid units, are also known and their bioactivity is also influenced by their pattern of glycosylation and other modification. The compounds so produced are particularly valuable because they include large numbers of compounds of known utility, for example as anthelminthics, insecticides, immunosuppressants, antifungal or antibacterial agents.

Streptomyces and closely-related genera of filamentous bacteria are abundant producers of polyketide metabolites. Although large numbers of therapeutically important polyketides have been identified, there remains a need to obtain novel polyketides that have enhanced properties or that possess completely novel bioactivity. The inexorable rise in the incidence of pathogenic organisms with resistance to antibiotics such as 14-membered macrolides or glycopeptides represents a significant threat to human and animal health. Current methods of obtaining novel polyketide metabolites include large-scale screening of naturally-occurring strains of *Streptomyces* and other organisms, either for direct production of useful molecules, or for the presence of enzymatic activities that can bioconvert an existing polyketide, which is added to the growth medium, into specific derivatives. These procedures are time-consuming and costly, and biotransformation using whole cells may in addition be limited by side-reactions or by a low concentration or activity of the intracellular enzyme responsible for the bioconversion. Given the complexity of bioactive polyketides, they are not readily amenable to total chemical synthesis in large scale. Chemical modification of existing polyketides has been widely used, but many desirable alterations are not readily achievable by this means.

Meanwhile, methods have been developed for the biosynthesis of altered polyketides and non-ribosomally-synthesised polypeptides by the engineering of the corresponding genes encoding the polyketide synthases and polypeptide synthetases respectively. The biosynthesis of polyketides is initiated by a group of chain-forming enzymes known as polyketide synthases. Two classes of polyketide synthase (PKS)

have been described in actinomycetes. One class, named Type I PKSs, represented by the PKSs for the macrolides erythromycin, oleandomycin, avermectin and rapamycin, consists of a different set or "extension module" of enzymes for each cycle of polyketide chain extension (Cortés, J. *et al.* Nature (1990) 348:176-178). The term "extension module" as used herein refers to the set of contiguous domains, from a β-ketoacyl-ACP synthase ("KS") domain to the next acyl carrier protein ("ACP") domain, which accomplishes one cycle of polyketide chain extension.

In-frame deletion of the DNA encoding part of the ketoreductase domain in module 5 of the erythromycin-producing PKS (also known as 6-deoxyerythronolide B synthase, DEBS) has been shown to lead to the formation of erythromycin analogues 5,6-dideoxy-3-α-mycarosyl-5-oxoerythronolide B, 5,6-dideoxy-5-oxoerythronolide B and 5,6-dideoxy, 6 β-epoxy-5-oxoerythronolide B (Donadio, S. *et al.* Science (1991) 252:675-679). Likewise, alteration of active site residues in the enoylreductase domain of module 4 in DEBS, by genetic engineering of the corresponding PKS-encoding DNA and its introduction into *Saccharopolyspora erythraea*, led to the production of 6,7-anhydroerythromycin C (Donadio, S. *et al.* Proc Natl. Acad. Sci. USA (1993) 90:7119-7123). WO 93/13663 describes additional types of genetic manipulation of the DEBS genes that are capable of producing altered polyketides. However many such attempts are reported to have been unproductive (Hutchinson, C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238, at p. 231).

WO 98/01546 describes the engineering of hybrid Type I PKS genes which utilise portions of PKS genes derived from more than one natural PKS, particularly derived from different organisms, and the use of such recombinant genes for the production of altered polyketide metabolites.

The second class of PKS, named Type II PKSs, is represented by the synthases for aromatic compounds. Type II PKSs contain only a single set of enzymatic activities for chain extension and these are re-used in successive cycles (Bibb, M. J. *et al.* EMBO J. (1989) 8:2727-2736; Sherman, D. H. *et al.* EMBO J. (1989) 8:2717-2725;

Fernandez-Moreno, M.A. *et al.* J. Biol. Chem. (1992) 267:19278-19290). The "extender" units for the Type II pKSs are usually acetate units, and the presence of specific cyclases dictates the preferred pathway for cyclisation of the completed chain into an aromatic product (Hutchinson, C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238). Hybrid polyketides have been obtained by the introduction of clones Type II PKS gene-containing DNA into another strain containing a different Type II PKS gene cluster, for example by introduction of DNA derived from the gene cluster for actinorhodin, a blue-pigmented polyketide from *Streptomyces coelicolor*, into an anthraquinone polyketide-producing strain of *Streptomyces galileus* (Bartel, P. L. *et al.* J. Bacteriol. (1990) 172:4816-4826).

The minimal number of domains required for polyketide chain extension on a Type II PKS when expressed in a *Streptomyces coelicolor* host cell (the "minimal PKS") has been defined for example in WO 95/08548 as containing the following three polypeptides which are products of the *act I* genes: first KS; secondly a polypeptide termed the CLF with end-to-end amino acid sequence similarity to the KS but in which the essential active site residue of the KS, namely a cysteine residue, is substituted either by a glutamine residue, or in the case of the PKS for a spore pigment such as the *whiE* gene product (Chater, K. F. and Davis, N. K. Mol. Microbiol. (1990) 4:1679-1691) by a glutamic acid residue; and finally an ACP. The CLF has been stated for example in WO 95/08548 to be a factor that determines the chain length of the polyketide chain that is produced by the minimal PKS. However, it has been found (Shen, B. *et al.* J. Am. Chem. Soc. (1995) 117:6811-6821) that when the CLF for the octaketide actinorhodin is used to replace the CLF for the decaketide tetracenomycin in host cells of *Streptomyces glaucescens*, the polyketide product is not found to be altered from a decaketide to an octaketide. An alternative nomenclature has been proposed in which KS is designated KSα and CLF is designated KSβ, to reflect this lack of confidence in the correct assignment of the function of CLF (Meurer, G. *et al.* Chemistry and Biology (1997) 4:433-443). International Patent Application WO 00/00618 has recently shown that CLF and its counterpart in Type I PKS multienzymes, the so-called KSQ domain, are involved in

initiation of polyketide chain synthesis. WO 95/08548 for example describes the replacement of actinorhodin PKS genes by heterologous DNA from other Type II PKS gene clusters, to obtain hybrid polyketides.

This ability to engineer PKS genes of both Type I and Type II raises the possibility of combinatorial biosynthesis of polyketides to produce diverse libraries of novel natural products which may be screened for desirable bioactivities. However, the aglycones produced by the recombinant PKS genes may be only partially, or not at all, processed by glycosyltransferases and other modifying enzymes into analogues of the mature polyketides. There is therefore an additional need to provide processes for efficient conversion of such novel aglycones into specific glycosylated products. Further, the invention of efficient processes for glycosylation would provide a new means to increase very significantly the diversity of combinatorial polyketide libraries, by utilisation of recombinant cells containing alternative cloned glycosyltransferases and alternative complements of activated sugars.

The well-known influence of glycosylation on biological activity has encouraged intensive research into the genes and enzymes governing the synthesis and attachment of specific sugar units to polyketide and polypeptide metabolites (for a review see Trefzer, A. et al. Natural Products Reports (1999) 16:283-299). Surveys of such metabolites have revealed a high diversity in the type of glycosyl substitution that is found, including a very large number of different deoxyhexoses and deoxyaminohexoses (see for a review Liu, H.-W. and Thorson, J.S.Annu. Rev. Microbiol. (1994) 48:223-256) review). The sequencing of biosynthetic gene clusters for numerous glycosylated polyketides and peptides has revealed the presence of such sugar biosynthetic genes, and also genes encoding the glycosyltransferases that transfer the glycosyl group from an activated form of the sugar, eg dTDP- or dUDP-forms, to the aglycone acceptor. For example, the *eryB* genes and the *eryC* genes of the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea* have been identified as involved in the biosynthesis and attachment of respectively, L-mycarose and D-desosamine to the aglycone precursor of erythromycin A (Dhillon, N. et al.,

Mol. Microbiol. (1989) 3:1405-1414; Haydock et al. Mol. Gen. Genet. (1991) 230:120-128; Salah-Bey, K. et al. Mol Gen. Genet (1998) 257:542-553; Gaisser, S. et al., Mol. Gen. Genet. (1998) 258:78-88; Gaisser, S. et al. (1997) Mol. Gen. Genet. 256: 239-251; Summers, D. et al. Microbiology (1997) 143: 3251-3262). Both WO 97/23630 and WO 99/05283 describe the preparation of an altered erythromycin by deletion of a specific sugar biosynthetic gene, so that an altered sugar becomes attached to the aglycone. Thus WO 99/05283 describes low but detectable levels of erythromycins in which for example desosamine is replaced by mycaminose (*eryCIV* knockout), or desmethylmycarosyl erythromycins (*eryBIII* knockout) are produced. Meanwhile methymycin analogues have been produced in which desosamine has been replaced by D-quinuvose (Borisova, S.A. et al. Org. Lett. (1999) 1:133-136), or through the incorporation of the *calH* gene of the calicheamycin gene cluster from *Micromonospora echinospora* into the methymycin producing strain (Zhao, L. et al. J. Amer. Chem. Soc. (1999) 121:9881-9882). Similarly, hybrid glycopeptides have been produced by using cloned glycosyltransferases from the vancomycin-producing *Amycolatopsis orientalis* to add D-xylose or D-glucose to aglycones of closely-related glycopeptides according to US Patent 5,871,983 (1999) (Solenberg, P. *et al.* Chem. Biol (1997) 4:195-202). Hybrid aromatic polyketides have also been produced, by interspecies complementation of a mutant individual sugar biosynthetic gene with a similar gene with a different stereospecificity. Thus instead of the natural daunosamine, 4'epi-daunosamine is produced in recombinant *Streptomyces peucetius* and attached by the daunosamine glycosyltransferase to the aglycone to yield the antitumour derivative epirubicin in place of doxorubicin (Madduri, K. et al. Nature Biotechnology (1998) 16:69-74). In all these cases, the specificity of the glycosyltransferase allowed the substitution of an alternative activated sugar, but the aglycone and glycosyltransferase were not heterologous to each other. It has been found that when oleandrose glycosyltransferase *oleG2* of *Streptomyces antibioticus* is cloned into the erythromycin-producing *Saccharopolyspora erythraea*, in addition to other products, the novel erythromycin in which cladinose/mycarose at C-3 is replaced by rhamnose, was obtained (Doumith, M. et al, Mol. Microbiol. (1999) 34:1039-1048). It was assumed that the activated rhamnose is produced by the host cells, and

is recruited by the *ole*G2 glycosyltransferase in competition with the activated mycarose known to be present.

Summary of the Invention

The present invention shows that cloned glycosyltransferases when rapidly screened for their ability to attach a range of activated sugars to a range of exogenously supplied or endogenously generated aglycone templates, show a surprising flexibility towards both aglycone and sugar substrates, and that this process allows the production of glycosylated polyketides in good yield. This overcomes the problem not only of supplying novel sugar attachments to individual polyketides, including polyketides altered by genetic engineering, but also of increasing the diversity of polyketide libraries by combinatorial attachment of sugars. It is particularly surprising that new glycosylated products can be produced in systems in which one or more of the components are heterologous to each other, the components being selected from the aglycone template, the sugar moiety or moieties, the glycosyltransferase, the host cell and/or genes encoding enzymes capable of modifying the sugar moiety, either before or after attachment to the aglycone template. In preferred embodiments, two, three, four or all of the components are heterologous to each other.

Accordingly, in a first aspect, the present invention provides a process for producing a hybrid glycosylated product by transferring one or more sugar moieties to an aglycone template, the process comprising:

transforming microorganism host cells with nucleic acid encoding a glycosyltransferase (GT); and,

providing an aglycone template to the GT so that the GT transfers one or more sugar moieties to the aglycone template to produce a hybrid glycosylated product;

wherein one or more of the sugar moiety or moieties, the aglycone template, the glycosyltransferase or the host cells are heterologous to the other components.

Preferably, the hybrid glycosylated product is other than compounds M1 to M4 disclosed in Doumith et al (*supra*), e.g. an erythromycin in which cladinose/mycarose

at C-3 is replaced by rhamnose.

In a further aspect, the present invention provides host cells transformed with nucleic acid encoding a glycosyltransferase (GT), wherein the GT is heterologous to the host cells and transfers one or more sugar moieties to an aglycone template within the cells to produce a hybrid glycosylated product.

In a further aspect, the present invention provides a process for producing a hybrid glycosylated product, the process comprising culturing the host cells defined above and isolating the product thus produced. In embodiments in which the aglycone template is supplied to the host cells, rather than being produced by the host cell, the process may comprise the additional step of supplying the aglycone template to the cells.

In further aspects, the present invention provides hybrid glycosylation products as obtainable by any of the processes disclosed herein.

In some embodiments of the present invention, a "hybrid glycosylated product" is one in which the aglycone template and the sugar moiety or moieties are heterologous to each other. In the processes described herein, one or more of the components of the system used to produce or modify the hybrid glycosylated product may be heterologous to one another. These components include the aglycone template, the sugar moiety or moieties, the microorganism strain/host cells, the glycosyltransferase which catalyses the attachment of the sugar moiety to the aglycone template and modifying genes capable of modifying the sugar moiety either before or after attachment to the aglycone template. The hybrid glycosylated product may also be subject of further processing or derivatisation, either by the strain or after isolating from culture medium.

In the present invention, an "aglycone template" is a polyketide, a peptide or a mixed polyketide-peptide which is capable of further processing, e.g. by a

glycosyltransferase, to transfer one or more activated sugar moieties to the template. The aglycone template may include forms of glycosylation other than that introduced by the heterologous GT. The cells may additionally contain one or more heterologous modifying genes, including but not limited to genes encoding enzymes or other proteins capable of carrying out methyl transfer, hydroxylation, or epoxidation reactions, on sugar moiety, before or after attachment to the aglycone template. Alternatively or additionally, further diversity in hybrid products can be obtained by deleting or modifying one or more homologous modifying genes.

In some embodiments, the aglycone template may be produced by the microorganism strain, either naturally or by transforming the strain with one or more genes or gene clusters capable of producing the template. By way of example, where a microorganism strain naturally produces a polyketide, the process may employ the polyketide aglycone template endogenously produced by the strain or the strain may be engineered to delete or inactivate the production of this template. In this latter case, the cells may be transformed with one or more PKS genes or a PKS gene cluster for the production of a heterologous template or, additionally or alternatively, one or more templates can be exogenously supplied to the host cells, e.g. in the screening method described below.

As mentioned above, the aglycone may be produced by the host cells by additionally cloning into the cell a recombinant polyketide synthase gene or genes either of type I or type II. The recombinant PKS genes may consist either of natural PKS genes or of mutated versions of natural PKS genes, or of hybrid PKS genes consisting of portions from at least two different natural type I PKS gene clusters; or natural type II PKS gene clusters, and may consist of a library of hybrid PKS genes of either type I or type II. Examples of PKS gene assemblies include those which produce the type I polyketide macrolides rifamycin, avermectin, rapamycin, immunomycin, or erythromycin, narbomycin, oleandomycin, pikromycin, spiramycin or tylosin; polyenes such as amphotericin B, candicidin, nystatin or pimaricin; polyethers such as monensin, salinomycin, semduramycin or tetronasin; and type II polyketides such as

actinorhodin, daunorubicin, oxytetracycline or tetracycline.

A preferred host cell strain is actinomycete, more preferably strains such as *Saccharopolyspora erythraea*, *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Streptomyces griseofuscus*, *Streptomyces cinnamonensis*, *Streptomyces fradiae*, *Streptomyces longisporoflavus*, *Streptomyces hygroscopicus*, *Micromonospora griseorubida*, *Streptomyces lasaliensis*, *Streptomyces venezuelae*, *Streptomyces antibioticus*, *Streptomyces lividans*, *Streptomyces rimosus*, *Streptomyces albus*, *Amycolatopsis mediterranei*, and *Streptomyces tsukubaensis*. Examples of preferred strains and preferred modifications to those strains to adapt them for use in the present invention are set out below.

Examples of glycosyltransferases suitable for use in accordance with the present invention (listing the GTs, their normal biosynthetic contexts and normal substrate specificities):

From the erythromycin pathway of *Saccharopolyspora erythraea*: desosaminyltransferase eryCIII and mycarosyltransferase eryBV.

From the megalomycin pathway of *Micromonospora megalomicea*: desosaminyltransferase megCIII, mycarosyltransferase megBV and megosaminyltransferase.

From the oleandomycin pathway of *Streptomyces antibioticus*: oleandrosyltransferase oleG2 (also transfers rhamnose and olivose) and desosaminyltransferase oleG1.

From the tylosin pathway of *Streptomyces fradiae*: mycaminosyltransferase tylMII deoxyallose transferase tylN and mycarosyltransferase tylCV.

From the midecamycin pathway of *Streptomyces mycarofaciens*: mycaminosyltransferase midI, deoxyallose transferase and mycarosyltransferase.

From the pikromycin/narbomycin pathway of *Streptomyces venezuelae*: desosaminyltransferase desVII.

From the spinosyn pathway of *Saccharopolyspora spinosa*: rhamnosyltransferase and forosaminyltransferase.

From the amphotericin pathway of *Streptomyces nodosus*: mycaminosyltransferase amphDI.

From the avermectin pathway of *Streptomyces avermitilis*: oleandrosyltransferase.

From the nystatin pathway of *Streptomyces*: mycaminosyltransferase.

From the polyene 67-121C pathway of *Actinoplanes caerulens*: mycosaminyltransferase, mannosyltransferase (transferring to the mycosamine).

From the elloramycin pathway of *Streptomyces olivaceaous Tü2353*: rhamnosyltransferase elmGT.

From the mithramycin pathway of *Streptomyces argillaceus*: olivosyltransferase mtmGIV.

From the daunomycin pathway of *Streptomyces peucetius*: daunosaminyltransferase dnrS.

From the urdamycin pathway of *Streptomyces fradiae* Tü2717: rhodinosyltransferase urdGT1c, olivosyltransferase urdGT1b, rhodinosyltransferase urdGT1a and olivosyltransferase urdGT2.

Preferably, the process further comprises the step of deleting or inactivating one or more genes in the microorganism host cells involved in the production of the aglycone

template and/or in its subsequent processing, thereby to suppress or alter the production of the natural aglycone template or product.

In a further aspect, the present invention provides a process for producing a library capable of producing a plurality of hybrid glycosylated products, the process comprising:

transforming microorganism host cells with nucleic acid encoding one or more glycosyltransferases (GT); and,

providing one or more aglycone templates to the GTs so that the GTs transfer one or more sugar moieties to the aglycone template to produce said plurality of hybrid glycosylated products;

wherein one or more of the sugar moiety or moieties, the aglycone template, the glycosyltransferase or the host cells are heterologous to the other components.

In further aspects, the present invention provides a process which further comprises screening the library for a hybrid glycosylated product having a desired characteristic.

In preferred embodiments, the library comprises 2 hybrid glycosylated products, more preferably at least 10 hybrid glycosylated products, more preferably at least 50 hybrid glycosylated products and still more preferably at least 100 hybrid glycosylated products.

In one embodiment, the present invention provides a process for screening for a hybrid glycosylated product, the process comprising:

producing one or more different microorganism host cells, each host cell being transformed with nucleic acid encoding a glycosyltransferase (GT), wherein the GT is heterologous to the microorganism strain, to form a library of host cells;

supplying the library with one or more aglycone templates;

screening the library for hybrid glycosylated products produced by the GTs transferring one or more sugar moieties to the aglycone templates.

Preferably, the processes described herein further comprise isolating a host cell producing a desired hybrid glycosylated product, and treating it further (e.g. culturing the cells and isolating the product produced) so that the product can be made in bulk. Preferably, in order to maximise diversity in the hybrid products, the screening method employs at least two different host cells and/or aglycone templates and/or glycosyltransferases and/or activated sugar moieties and/or heterologous modifying genes capable of modifying the sugar moiety before or after transfer to the aglycone template, more preferably at least 3, more preferably at least 5, more preferably at least 10, more preferably at least 20 and most preferably at least 50 different cells and/or templates.

This screening method allows a large number of novel hybrid products to be generated and screened maximising the number and variety of products that can be made and tested. Desired hybrid products can be detected by their biological activity (e.g. as antibiotics).

It will be evident to those skilled in the art that production of hybrid glycosides may be done in a number of alternative ways using the present invention, e.g.:

(1) by including all the required genes in the same cell, whether introduced separately or as a single cassette; or

(2) stepwise, the product of one fermentation with a recombinant cell containing some of the expressed genes (either after purification or used as a filtered supernatant) in its turn being fed to a second bioconversion strain containing the rest of the expressed genes.

In the latter case, the process of the invention may comprise the steps of:

producing one of the aglycone template or the sugar moiety in first host cells as a first product;

optionally, purifying the first product from a culture of the first host cells; and,

adding the first product to a second host cell comprising one or more genes encoding the other of the aglycone template or the sugar moiety and one or more

glycosyltransferases, so that the sugar moiety is transferred to the glycosyltransferase, to produce the hybrid glycosylated product.

The first product may be in various degrees of purification, i.e. it may be employed as a filtered supernatant, in an isolated form or in any degree of purification compatible with production in the second host cells.

In some embodiments, the host cells may additionally contain cloned and expressed genes for sugar biosynthesis, either an entire group of genes needed to furnish a naturally occurring or novel deoxysugar, for example all the specific *eryB* genes from *Saccharopolyspora erythraea* or elsewhere required to make activated dTDP-mycarose from metabolic intermediates common to actinomycete cells, or such gene sets missing certain genes so that altered activated sugars are provided, or certain individual genes only that modify the type of activated sugars produced by endogenous deoxyhexose biosynthetic pathways. Thus, the present invention includes the possibility of transforming the host cells with one or more genes for modifying the aglycone template, e.g. to provide alternative positions at which glycosylation can be introduced, to modify existing functionality in the template or which are involved in the downstream processing of the hybrid glycosylation products.

The cells may be additionally cultivated in the presence of cerulenin, which specifically suppresses endogenous polyketide biosynthesis, or additionally or alternatively mutated to delete or otherwise inactivate one or more of the PKS genes naturally present within the cells, in either case the result is to decrease competition with the supplied aglycone.

In a further aspect, the present invention provides the hybrid glycosylated products produced by any one of the processes described herein.

In a further aspect, the present invention provides novel hybrid glycosylated products resulting from the attachment of sugar moieties to aglycone templates. Examples of

hybrid products include those comprising:

(a) one or more natural sugars linked to an erythronolide at the 7-position.

(b) one or more rhamnose or substituted (e.g. methyl) rhamnose sugars linked to an erythronolide

(c) one or more mycarose or substituted mycarose sugars linked to an erythronolide;

and combinations of (a), (b) and (c) sugar substituents on an erythronolide (e.g. erythronolide B).

Examples of hybrid products based on an erythromycin template include those comprising:

(a) one or more natural sugars linked to erythromycin at the 7-position;

(b) one or more mycarose or substituted mycarose sugars linked to an erythromycin;

(c) one or more mycaminose or substituted mycaminose sugars linked to an erythromycin;

and combinations of (a), (b) and (c) sugar substituents on an erythromycin (e.g. erythromycin A).

Examples of hybrid products based on an tylactone template include those comprising:

(a) one or more glucose or substituted glucose sugars linked to a tylactone;

(b) one or more desosaminose or substituted desosaminose sugars linked to a tylactone;

(c) one or more mycaminose or substituted mycaminose sugars linked to a tylactone;

(d) one or more rhamnose or substituted rhamnose sugars linked to a tylactone;

and combinations of (a), (b), (c) and (d) sugars substituents such as a rhamnose and a mycaminose sugar linked to a tylactone (e.g. 23-O-rhamnosyl 5-O-mycaminosyl tylactone).

Specific examples of hybrid products of the invention are:

3-O-(2'-O-methylrhamnosyl)erythronolide B

3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B

3-O-(2',3',4'-tris-O-methylrhamnosyl)erythronolide B

3-O-mycarosyl-erythronolide B

8a-hydroxy-3-O-mycarosyl erythronolide B

8,8a-epoxy-3-O-mycarosyl erythronolide B

8,8a-dehydro-6-deoxyerythronolide B

8-hydroxy-6-deoxyerythronolide B

3-O-(2'-O-methylrhamnosyl)erythromycin D

3-O-(2',3'-bis-O-methylrhamnosyl)erythromycin D

3-O-(2',3',4'-tris-O-methyl rhamnosyl)erythromycin D

5-O-mycaminosyl-erythromycin A

5-O-mycaminosyl-4"-O-mycarosyl erythromycin A

5-O-glucosyl-tylactone

5-O-desosaminyl-tylactone

23-O-rhamnosyl 5-O-mycaminosyl tylactone

5-O(2'-O)- bis-glucosyl-tylactone

3-O-rhamnosyl-8,8a-dehydro-6-deoxyerythronolide B

3-O-rhamnosyl-8,8a-dihydroxy-erythronolide B

3,5 di-O-mycarosyl erythronolide B.

In a further aspect, the present invention provides a process for assembling a gene set in a cassette for transformation of a host cell for carrying out the processes described herein. The strategy to prepare gene cassettes with different combinations of glycosyltransferase- and methyltransferase genes is adapted from a technique previously described (WO 00/77181A2) to build gene cassettes expressed under the control of the *actII*-Orf4 regulator. The expression of these gene cassettes in a suitable strain background is a powerful approach to generate novel post-PKS modified polyketides in a random or directed fashion. The method is based upon the introduction of *XbaI* restriction sites at the 3' and 5'- end of the PCR fragments. The

introduction of a *XbaI* site at the 5'- end of the PCR fragment which is sensitive to the Dam methylase of the strain background will protect this site from further *XbaI* digest. To retain the Shine Dalgarno sequence 5' of the respective gene the pSG142 derived constructs which contain these genes were used as a template. Using plasmid DNA isolated from dam⁻ host strains (such as E. coli ET 12567 - McNeil et al, (1992) Gene, 111, 61-68), the amplified genes can be isolated as *XbaI* fragments. Using a host strain with an active Dam methylase such as E. coli DH10B these fragments can be sequentially cloned into gene cassettes. This technique provides the means to build gene cassettes of different length and different order using the same strategy over and over again. An overview of the strategy described here and the isolated gene cassettes is depicted in Fig. 18. In some cases, expression of the terminal gene surprisingly was increased when nucleic acid encoding a histidine tag was fused to the 3' end of the gene cassette.

Where more than one gene is required to be introduced and expressed in the said host cells, many ways will readily occur to the person skilled in the art as to how this goal may be achieved in a way that ensures that there will be coordinated expression of all the required gene products. However, the present invention further provides a novel process and expression cassette for achieving this goal which, in one embodiment, allows the stepwise contiguous head to tail assembly of individual sugar pathway genes, or of heterologous modifying genes, or of both types of gene, and thereby not only places them on a single region of DNA under the control of a common promoter, but also thereby facilitates their further genetic manipulation together as a single unit or cassette. Each gene to form part of such a cassette assembly can be either a natural or modified gene, or synthetic versions of a natural genes, and such natural genes may be obtained either from the said host cells or may be heterologous to the said host cells.

Accordingly, in a still further aspect, the present invention provides an expression cassette comprising one or more glycosyltransferase genes and one or more auxiliary genes, operably linked under the control of a promoter. As described above, the

auxiliary genes may be genes encoding proteins involved in the biosynthesis of one or more sugars (a 'sugar pathway gene') to enable a host cell transformed with the expression cassette to produce one or more sugar moieties for subsequent transfer to an aglycone template. Other examples of heterologous auxiliary genes include enzymes involved in the processing of sugar moieties or the aglycone template, either before or after the sugar moiety is transferred to the aglycone by the GT. Examples of these enzymes include methyltransferases and P450s which are responsible for hydroxylation of the aglycone template. Preferably, the genes in the cassette are under the control of a single, preferably strong, promoter. Further, the present inventors have found that by incorporating a nucleic acid sequence encoding a histidine tag adjacent to (or immediately 3') the terminal gene in the expression cassette so that expression of genes in the cassette which are distal to the promoter is improved.

In a further aspect, the present invention provides a process of producing an expression cassette comprising one or more glycosyltransferase genes and one or more auxiliary genes, the process comprising operably linking the genes together under the control of a promoter. The process may comprise the further step of transforming a host cell with the expression cassette and expressing the genes comprised within it to produce the GT and proteins encoded by the auxiliary genes.

In a further aspect, the present invention provides a host cell transformed with such an expression cassette.

Embodiments of the present invention will now be described by way of example and not limitation with reference to the accompanying figures.

Brief Description of the Figures

Figure 1A: Scheme for the isolation of *S. erythaea* Δorf14 containing a 1247-bp deletion in *eryB*:. *tsr*, thiostrepton resistance.

Figure 1B: Scheme for the isolation of *S. erythaea* strain DM containing both a

Fig.12: Analysis of the culture supernatants of SGT2 and SGT2pSGSpnK after feeding with 3-O-(2'-O-methylrhamnosyl)erythronolide B.

Fig. 13: Overview of the plasmids pSGSpnHK and pSGSpnHKI.

Fig. 14: Structure of 3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B.

Fig. 15: Structure of 3-O-(2',3',4'-tris-O-methylrhamnosyl)erythronolide B.

Fig. 16: Structures of 3-O-rhamnosyl-erythromycin D (A); 3-O-(2'-O-methylrhamnosyl) erythromycin D (B); and 3-O-(2',3'-bis-O-methylrhamnosyl)erythromycin D (C).

Fig. 17: Bioassay of 3-O-(2'-O-methylrhamnosyl)erythromycin D and erythromycin A.

Fig. 18: Strategy to isolate gene cassettes of *oleG2*, *spnI*, *spnH*, *spnK* and *eryCIII*.

Fig. 19: Results of the analysis of the culture supernatant of SGT3pSGcas*oleG2spnIspnK*.

Fig. 20: Results of the analysis of the culture supernatant of SGT3pSGcas*oleG2spnIspnKeryCIII*.

Figure 21: Structures of 8a-hydroxy-3-O-mycarosyl erythronolide B and 8,8a-epoxy-3-O-mycarosyl erythronolide B.

Figure 22: Structures of 8,8a-dehydro-6-deoxyerythronolide B and 8-hydroxy-6-deoxyerythronolide B.

Figure 23: Scheme for the construction of gene cassettes and transformation into *S. erythraea*.

Fig.12: Analysis of the culture supernatants of SGT2 and SGT2pSGSpnK after feeding with 3-O-(2'-O-methylrhamnosyl)erythronolide B.

Fig. 13: Overview of the plasmids pSGSpnHK and pSGSpnHKI.

Fig. 14: Structure of 3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B.

Fig. 15: Structure of 3-O-(2',3',4'-tris-O-methylrhamnosyl)erythronolide B.

Fig. 16: Structures of 3-O-rhamnosyl-erythromycin D (A); 3-O-(2'-O-methylrhamnosyl) erythromycin D (B); and 3-O-(2',3'-bis-O-methylrhamnosyl)erythromycin D (C).

Fig. 17: Bioassay of 3-O-(2'-O-methylrhamnosyl)erythromycin D and erythromycin A.

Fig. 18: Strategy to isolate gene cassettes of *oleG2*, *spnI*, *spnH*, *spnK* and *eryCIII*.

Fig. 19: Results of the analysis of the culture supernatant of SGT3pSGcas*oleG2spnIspnK*.

Fig. 20: Results of the analysis of the culture supernatant of SGT3pSGcas*oleG2spnIspnKeryCIII*.

Figure 21: Structures of 8a-hydroxy-3-O-mycarosyl erythronolide B and 8,8a-epoxy-3-O-mycarosyl erythronolide B.

Figure 22: Structures of 8,8a-dehydro-6-deoxyerythronolide B and 8-hydroxy-6-deoxyerythronolide B.

Figure 23: Scheme for the construction of gene cassettes and transformation into *S. erythraea*.

Figure 24: Structures of 3-O-rhamnosyl-8,8a-dehydro-6-deoxyerythronolide B and 3-O-rhamnosyl-8,8a dihydroxy-6-deoxyerythronolide B.

Figure 25: DNA sequence of the *oleG1* start region (accession number AJ002638).

Figure 26: Results of the complementation of the *eryCIII* mutation in *S. erythraea* SGT2 and SGT2pSGOleG1. 3-O-mycarosyl erythronolide B was fed to the cultures as described previously (Gaisser et al., 2000). The presence of 3-O-mycarosyl erythronolide B is indicated by a peak of a retention time of 20 min. The peak of a retention time of 15.7 and 734 m/z indicates the presence of erythromycin A.

Figure 27: Results of the feeding of 6-deoxyerythronolide B to SGQ1, SGQ1pSGOleG1, SGQ1pSGOleG2 and SGQ1pSGOleP. Structures of the major compounds are indicated.

Figure 28: Results of the feeding of 3-O-rhamnosyl-6-deoxyerythronolide B to SGQ1, SGQ1pSGOleG1, SGQ1pSGOleG2 and SGQ1pSGOleP. Structures of the major compounds are indicated.

Figure 29: Structure of 23-O-rhamnosyl-5-O-mycaminosyl-tylactone.

Figure 30: Structure of 23-O-rhamnosyl-5-O-desosaminyl-tylactone.

Figure 31: Structure of 5-O-(2'-O-)bis-glucosyl-tylactone.

Figure 32: Structures of 3-O-mycarosyl-5-O-mycaminosyl-erythromycin A and 3-O-mycarosyl-5-O-mycaminosyl-4"-O-mycarosyl-erythromycin A.

Figure 33: Sequence of the genes *tylMI, tylM3* and *tylB*, showing 8 amino acid changes in *tylB* as compared to the published sequence.

Figure 34: Assembly of gene cassettes in pUC18 to produce constructs pUC18tyl*MIII-tylB* and pUC18*tylMIII-tylB-tylMI.*

Figure 35: Restriction maps for pSGCIII, pSGTYLM2, pSGDESVII and pSGTYLCV.

Figure 36: Restriction map of plasmid pGG1.

Figure 37: Mass spectra of novel compounds 3-O-mycarosyl-5-O-mycaminosyl-erythromycin A and 3,5-di-O-mycarosyl-erythronolide B.

Materials and Methods

Escherichia coli XL1-Blue MR (Stratagene) and *E. coli* DH10B (GibcoBRL) were grown in 2xTY medium as described by Sambrook *et al.* (Molecular Cloning, A Laboratory Manual, 2nd Edition (1989) Cold Spring Harbor, NY, Cold Spring Harbor Press). Vectors Litmus28 and pUC18 were obtained from New England Biolabs and vector pQE-16 from QIAGEN. Vector pIJ702 (Katz *et al.* J. Gen. Microbiol. (1983) 129:2703-2714) was kindly provided by D. A. Hopwood (John Innes Institute, Norwich, UK). Vector pUC2G which contains a 6.2 kb *BglII* fragment from cosAB35 cloned into pUC18 was kindly provided by J. Salas (University of Oviedo, Spain). Vector pLQD1 which contains a 5076 bp PvuII fragment from cosmid cos25G8 cloned into pUC18 was kindly provided by J. Salas (University of Oviedo, Spain).

Cosmid no 7 which has been isolated from a *Streptomyces fradiae* cosmid library, was kindly provided by J. Cortés. *E. coli* transformants were selected with 100 μg/ml ampicillin. The *Saccharopolyspora erythraea* NRRL 2338-red variant strain (Hessler *et al.*, Appl. Microbiol. Biotechnol. (1997) 47:398-404) was kindly provided by J.M. Weber and was routinely maintained on M1-102 agar (Kaneda *et al.* J. Biol. Chem. (1962) 237:322-327), R2T20 (Yamamoto *et al.* J. Antibiot. (1986) 34:1304-1313), R2T2 (same as R2T (Weber *et al.* J. Bacterial. (1985)164:425-433), but without peptone), and TSB (Difco) for liquid cultures at 30°C. *Bacillus subtilis* ATCC 6633 was used in bioassays to assess erythromycin production as described (Gaisser *et al.*

(1997), *supra*). These assays were modified to investigate erythromycin production after feeding with erythronolide B or 3-α-mycarosyl-erythronolide B kindly provided by J.-M. Michel (Hoechst Marion Roussel, Romainville, France). Both metabolites (10 μl of 10mM stock solution) were applied to agar wells cut into the *S. erythraea* lawn and incubated at 30°C overnight as described (Gaisser *et al.* (1997) *supra*) and the development of zones of inhibition in the *B. subtilis* lawn around *S. erythraea* colonies was assessed. Expression vectors in *S. erythraea* were derived from plasmid pCJR24 (Rowe *et al.*, Gene (1998) 216:215-223). Plasmid-containing *S. erythraea* were selected with 25 μg/ml thiostrepton. To investigate the production of antibiotics, *S. erythraea* strains were grown in sucrose-succinate medium (Caffrey *et al.* (1992) FEBS Lett. 304:225-228) as described (Gaisser *et al.*, (1997) *supra*) and the cells were harvested by centrifugation. Tylactone was kindly provided by B. Wilkinson of Glaxo Group Research, Stevenage, UK. 150 μl of a 100 mg/ml stock solution was added to a 500 ml *S. erythraea* expression culture.

DNA manipulation and sequencing

DNA manipulations, PCR and electroporation procedures were carried out as described in Sambrook *et al.* (1989) *supra*. Protoplast formation and transformation procedures of *S. erythraea* were as described (Gaisser *et al.*, (1997) *supra*). Southern hybridizations were carried out with probes labelled with digoxigenin using the DIG DNA labelling kit (Boehringer Mannheim). DNA sequencing was performed by the method of Sanger *et al.* (P.N.A.S. USA (1977) 74:5463-5467), using automated DNA sequencing on double stranded DNA templates with an Applied Biosystems 373A sequencer. Sequence data were analysed using the Staden Programs (Staden, R. Nucl. Acids Res. (1984) 12:521-528) and the Genetics Computer Group (GCG, version 10) software package (Devereux *et al.*, Nucl. Acids Res. (1984) 12:387-395).

Extraction and mass spectrometry

10 ml of each fermentation broth was centrifuged and the pH of the supernatant was adjusted to pH 9. The supernatant was extracted twice with an equal volume of ethyl acetate. The organic layer was dried over Na_2SO_4, evaporated to dryness and then

redissolved in 0.3 ml acetonitrile / water (1:1 v/v). Mass spectrometry was performed on a BioQ (Micromass, Manchester, UK) or a Finnigan LCQ (Finnigan, CA) instrument. High resolution spectra were obtained on a Bruker BioApex II FT-ICR (Bruker, Bremen, FRG).

For NMR analysis, the bacterial broth was centrifuged and the pH of the supernatant was adjusted to about pH 9. The supernatant was extracted with three equal volumes of ethyl acetate, the extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure to yield a yellow solid. Final purification was achieved using reversed phase preparative HPLC on a Gilson 315 System using a 21 mm x 250 mm Prodigy ODS3 column (Phenomenex, Macclesfield, UK.). The mobile phase was pumped at a flow rate of 21 ml/min as a binary system consisting of 45% CH_3CN, 55% 20 mM NH_4OAc [pH 5.5 with HCOOH] increasing linearly to 95% CH_3CN over 25 min.

^1H Nuclear Magnetic Resonance (NMR) spectra were acquired at 800 MHz on a Bruker Avance DRX800 and at 500 MHz on a Bruker Avance DRX500. ^{13}C NMR spectra were acquired at 100 MHz on a Bruker Avance DRX400 spectrometer. Samples for NMR analysis were dissolved in CD_3OD and the experiments were performed at 300 K. High Performance Liquid Chromatography (HPLC) was performed on a Hewlett Packard HP1100 liquid chromatograph. Liquid Chromatography Mass Spectrometry (LC-MS), Tandem Mass Spectrometry (MS/MS) and MS^n spectra were obtained on a Finnigan MAT (San Jose, CA) LCQ. High resolution Quadrupole Time Of Flight MS/MS data were obtained on a Micromass (Macclesfield, U.K.) QTOF. High resolution MS^n were obtained on a Bruker Daltonics BioApex II 4.7 T Fourier Transform Ion Cyclotron Resonance mass spectrometer using PEG as external calibrant.

Chromosomal deletion of eryBV (Fig. 1A)

Vector pNCO28 (Gaisser et al, 1997) (Fig. 1A) was digested with *XbaI/NheI* and *XbaI/SpeI*, a 3.8 kb band and a 0.4 kb fragment were isolated, ligated and transformed

into *E.coli* DH10B. A vector construct pNCOΔ containing the deletion of *eryBV* was isolated and the *SpeI/NheI* ligation site was verified by sequencing. After digestion with *BglII*, the plasmid was ligated with *BglII*-cut pIJ702 and used to transform *E. coli* DH10B. Plasmid pNCOΔpIJ702 was isolated and used to transform *S. erythraea* NRRL 2338 (red variant). Colonies were selected for thiostrepton resistance and integration into the chromosome was confirmed by Southern analysis. To allow for the second recombination event, integrants were subcultured at least four times in TSB medium (Difco) at 30°C. Single colonies were screened for thiostrepton sensitivity and for erythromycin production. The 1247 nt chromosomal deletion of *eryBV* was verified by using the 1.5 kb *NcoI* fragment of plasmid pNCOΔ to probe *ClaI/PstI*- and *NcoI*-digested chromosomal DNA of the wild type strain and of mutant Δorf14. Analysis of the wild type *S. erythraea* showed the expected 2.9 kb *ClaI/PstI* and 2.7 kb *NcoI* band after hybridization. When chromosomal DNA of Δorf14 was treated similarly, only a 1.6 kb *ClaI/PstI* and a 1.5 kb *NcoI* fragment were detected indicating that *eryBV* had been removed. The mutant Δorf14 was tested in a bioassay. No zones of inhibition were observed around the *S. erythraea* colonies in the *B. subtilis* lawn, indicating that no erythromycin A is produced by the mutant strain.

Chromosomal deletion of eryCIII (Fig. 1B)

Plasmid λSE55 (Haydock *et al.*, Mol. Gen. Genet. (1991) 230:120-128) was digested with *NcoI/XhoI* (Fig. 1B) and a 2.4 kb fragment was cloned into *NcoI/XhoI* digested vector Litmus28. Plasmid pLitNX was isolated and its identity verified by restriction digestion and by sequencing. Using λSE55 as a template and primers SG10 5'-GGCGATGTGCCAGCCCGCGAAGTT -3' and SG11 5'-AGCCGTCACCGGCCATGGTCGTCGGCATCT -3', a 573 nt fragment was amplified using PCR, treated with T4 polynucleotide kinase and cloned into *SmaI*-cut pUC18. The sequence was checked, and this plasmid construct was then digested with *NcoI* and a 0.5 kb fragment was isolated, ligated into *NcoI*-digested pLitNX and used to transform *E. coli* DH10B. Plasmid pLitNXP was isolated and the correct insert was verified, by restriction digestion and sequencing, as bearing a 1191 nt deletion in *eryCIII*. The construct pLitNXP was digested with *BglII* and ligated into

pIJ702 previously treated with *Bgl*II, and the mixture was used to transform *E. coli* DH10B. Plasmid pLitNXPIJ was isolated and used to transform the *S. erythraea* mutant Δorf14 as described. Integration was verified by Southern blot hybridization. After subculturing, single colonies were screened for thiostrepton sensitivity as described above. Thiostrepton sensitive colonies were grown in small patches and agar plugs taken from well-grown areas were placed on bioassay plates containing 3 alpha-mycarosyl-erythronolide B. No inhibition zone was observed around colonies of the isolated *S. erythraea* strain DM (Δorf14Δorf8). The 1247 nt chromosomal deletion was verified in Southern blot hybridizations as described for the mutant Δorf14. The 1191 nt chromosomal *eryCIII* deletion was verified using a digoxygenin-labelled 573 nt DNA fragment, amplified by primers SG10 and SG11, to probe *Nco*I- and *Cla*I/*Bgl*II-digested chromosomal DNA of the wild type strain and of the mutant DM (Δorf14Δorf8). Analysis of the wild type *S. erythraea* showed the expected 1.7 kb *Nco*I and 12.4 kb *Bgl*II bands after hybridization. When chromosomal DNA of DM was treated similarly, only an 11 kb *Bgl*II and a 0.5 kb *Nco*I fragment were detected indicating that 1.2 kb of the *eryCIII* gene had been removed.

Chromosomal deletion of eryA

To allow the feeding of different aglycones to a *S. erythraea* mutant strain housing heterologous glycosyltransferase genes, the *eryA* deletion previously described for JC2/Del60 (Rowe *et al.*, (1998) *supra*) was also introduced into the *S. erythraea* mutant strain DM. *S. erythraea* SGT2 was isolated (Fig. 2) and all three deletions (*ΔeryAΔBVΔCIII*) were verified by Southern blot analysis. Culture broths of *S. erythraea* SGT2 were analysed by electrospray mass spectrometry. No peaks corresponding to either erythromycin A or precursor metabolites were found.

Construction of expression plasmid for eryBV

The gene *eryBV* was amplified by PCR using the primers 1518 5'-GGGGGATCCCATATGCGGGTACTGCTGACGTCCTTCG -3' and 1519 5'-GAAAAGATCTGCCGGCGTGGCGGCGCGTGAGTTCCTC -3', which introduce a

*Bam*HI and a *Nde*I site at the 5' end and a *Bgl*II site at 3' end of *eryBV*. After treatment with T4 polynucleotide kinase the PCR product was cloned into *Sma*I-cut pUC18 and the resulting plasmid used to transform *E. coli* DH10B. The sequence of *eryBV* in the isolated plasmid was checked, and this plasmid was then digested using the restriction enzymes *Bam*HI and *Bgl*II, a 1.2 kb fragment was isolated and ligated into the identically-digested vector fragment of pQE-16, which introduced a C-terminal His_6-tag into EryBV. The pQE-16 derived plasmid was digested with *Nde*I and *Xba*I and a 2.2 kb fragment was isolated and ligated into vector pCJR24 previously cut with *Nde*I and *Xba*I, to give plasmid pSG2414. To allow the recombination of this plasmid into the genome of *S. erythraea*, a 1.7 kb *Nco*I fragment from cosmid cos6B (Gaisser *et al*., (1997) *supra*) from the *ermE* distal flank of the erythromycin biosynthetic cluster (Pereda *et al*., Gene (1997) 193:65-71) was isolated and cloned into the pQE-16 derived *Nco*I site. This final construct was named pSG142 .

Construction of expression plasmid for eryCIII

For expression of *eryCIII*, primers SG14 5'-GAAAAGATCTTCGTGGTTCTCTCCTTCCTGCGGCCAG -3' and SG15 5'-GGGGGATCCCATATGCGCGTCGTCTTCTCCTCCAT- 3' were used to amplify *eryCIII* with λSE55 as template. The 1287 bp DNA fragment was isolated, treated with T4 polynucleotide kinase and cloned into *Sma*I cut pUC18. After transformation into *E. coli* DH10B the construct was isolated and the sequence of *eryCIII* was verified. After digestion with *NdeI/BglII*, a 1.2 kb fragment was isolated, ligated with the vector fragment of *NdeI/BglII* digested pSG142 and used to transform *E. coli* DH10B. Plasmid pSGCIII was isolated.

Construction of expression plasmid for oleG2

For expression of *oleG2*, the primers Ole3 5'-GGCGGATCCCATATGCGCGTACTGCTGACCTGCTTCGCC -3' and Ole4 5'-CCAGATCTGCCCGCATGGTTCCCGCCTCCTCGTCC -3' were used to amplify *oleG2* using plasmid pUC2G or chromosomal DNA of *Streptomyces antibioticus* as a

template. The PCR fragment was isolated, treated with T4 polynucleotide kinase and cloned into *Sma*I cut pUC18. After transformation into *E. coli* DH10B the construct was isolated and the sequence of *oleG2* was verified. After digestion with *NdeI/BglII*, a 1.3 kb fragment was isolated, ligated with the vector fragment of *NdeI/BglII* digested pSG142 and used to transform *E. coli* DH10B. Plasmid pSGOLEG2 was isolated.

Construction of expression plasmid for tylM2

For expression of *tylM2* (Gandecha *et al.*, Gene (1997) 184:197-203) the primers Tyl1 5'- GTGGAGATCTCCTTTCCGGCGCGGATCGGGACCG -3' and Tyl2 5'- GGGGGATCCCATATGCGGGTACTGCTGACCTGTATCG -3' were used to amplify *tylM2* using cosmid no 7 as a template or chromosomal DNA of *Streptomyces fradiae*. Primer Tyl2 was chosen on the basis of sequence comparisons of known glycosyltransferases, which indicated the methionine at position 21 of the published sequence (accession no X81885) to be the start codon. The changes to the DNA sequence of *tylM2* noted in a recent update (X81885/June 1999) were confirmed independently in this work. The PCR DNA fragment was isolated, treated with T4 polynucleotide kinase and cloned into *Sma*I cut pUC18. After transformation into *E. coli* DH10B the construct was isolated and the sequence of *tylM2* was verified. After digestion with *NdeI/BglII*, a 1.3 kb fragment was isolated, ligated with the vector fragment of *NdeI/BglII* digested pSG142 and used to transform *E. coli* DH10B. Plasmid pSGTYLM2 was isolated.

Construction of expression plasmid for desVII

For expression of *desVII* (Xue *et al.*, (1998) P.N.A.S. USA, 95:12111-12116) the primers Pik1 5'- GGAGGATCCCATATGCGCGTCCTGCTGACCTCGTTCG-3' and Pik2 5'- GGGGTGCAGATCTGTGCCGGGCGTCGGCCGGCGGG-3' were used to amplify *desVII* using genomic DNA of *Streptomyces venezuelae* as a template. The PCR DNA fragment was isolated, treated with T4 polynucleotide kinase and cloned into *Sma*I cut pUC18. After transformation into *E. coli* DH10B the construct was isolated and the sequence of *desVII* was verified. After digestion with *NdeI/BglII*, a 1.3 kb fragment was isolated, ligated with the vector fragment of

*NdeI/Bgl*II digested pSG142 and used to transform *E. coli* DH10B. Plasmid pSGDESVII was isolated.

Construction of expression plasmid for tylH

For expression of *tylH* (Fouces *et al.*, Microbiology, (1999) 145:855-868) the primers TylH1 5'- CCGCCCGGCCCAGATCTCCGCGGCCCTCATGCGT-3' and TylH2 5'-TTGAGGCCGCAGCGACATATGTCCTCGTCCGGGGA-3' were used to amplify *tylH* using genomic DNA of *Streptomyces fradiae* as a template. The PCR DNA fragment was isolated, treated with T4 polynucleotide kinase and cloned into *Sma*I cut pUC18. After transformation into *E. coli* DH10B the construct was isolated. After digestion with *NdeI/Bgl*II, a 1.6 kb fragment was isolated, ligated with the vector fragment of *NdeI/Bgl*II digested pSG142 and used to transform *E. coli* DH10B. Plasmid pSGTYLH1 was isolated.

Construction of expression plasmid for tylN

For expression of *tylN* (Fouces *et al.*, 1999) the primers Tyl5 5'-GGGCATATGCGCATAGCGTTGCTGACCATGGGCT -3' and Tyl4 5'-GGCCAGATCTGCCGGGGGTGTGTGCCGTGGTCCGGG-3' were used to amplify *tylN* using genomic DNA of *Streptomyces fradiae* as a template. The PCR DNA fragment was isolated, treated with T4 polynucleotide kinase and cloned into *Sma*I cut pUC18. After transformation into *E. coli* DH10B the construct was isolated. After digestion with *NdeI/Bgl*II, a 1.3 kb fragment was isolated, ligated with the vector fragment of *NdeI/Bgl*II digested pSG142 and used to transform *E. coli* DH10B. Plasmid pSGTYLN was isolated.

Construction of expression plasmids for both tylH and tylN

Plasmid pSGTYLH was digested with *Bgl*II and pSGTylN was digested with *AflII/Nhe*I. The fragments were submitted to fill-in reactions using Klenow polymerase (Sambrook *et al.*, (1989) *supra*). The pSGTYLH vector derived DNA was isolated and ligated with the 1.5 kb fragment encoding TylN. *E. coli* DH10B was transformed with the ligation mixture. Plasmid pSGTYLHN was isolated.

Construction of expression plasmid for oleD

For expression of *oleD* (Hernandez *et al.*, Gene (1993) 134:139-140) the primers OleD1 5'-CCGGATCCCATATGACCACCCAGACCACTCCCGCCCACATC -3' and OLED2 5'- CGAGATCTCAAAGCGGATCTCTGCCGGTCGGAACGGA-3' were used to amplify *oleD* using pLQD1 (Luis M. Quiros) or chromosomal DNA of *Streptomyces antibioticus* as a template. The PCR DNA fragment was isolated, treated with T4 polynucleotide kinase and cloned into *Sma*I cut pUC18. After transformation into *E. coli* DH10B the construct was isolated. After digestion with *Nde*I/*Bgl*II, a 1.3 kb fragment was isolated, ligated with the vector fragment of *Nde*I/*Bgl*II digested pSG142 and used to transform *E. coli* DH10B. Plasmid pSGOLED was isolated.

Isolation of 3-O-rhamnosyl-erythronolide B and 3-O-rhamnosyl-6-deoxy-erythronolide B

The plasmid pSGOLEG2 was transformed into *S. erythraea* mutant DM cells and culture broths of the transformed strains were analysed as described by Gaisser et al. (1997) and by Gaisser et al. (1998). Analysis of the S. erythraea mutant DM(pSGOLEG2) by electrospray mass spectroscopy revealed the presence of only two distinct new peaks, at m/z 555 and 571 respectively, which were not present in the culture broth of the *S. erythraea* strain DM examined under the same conditions of growth, extraction and analysis. MS/MS experiments revealed that the ion with m/z of 571 fragmented into an ion with m/z of 425 (corresponding to the sodium salt of erythronolide B, EB-Na+) corresponding to the loss of m/z 146 (rhamnose). The fragmentation pattern of the ion of m/z 555 was identical to that of the fragment of m/z 571, but shifted lower by 16 mass units, indicating a missing hydroxy group. This was evidence that the compound with m/z 555 represents rhamnosyl-6-deoxyerythronolide B. To confirm these structures, 1.5 litres of culture broth were used to purify 4.6 mg of 3-O-rhamnosyl-erythronolide B and 2.7 mg of 3-O-rhamnosyl-6-deoxyerythronolide B. Both compounds were analysed and the structures were fully confirmed by ^{1}H and ^{13}C NMR.

Table 1: ^{1}H NMR Data for 3-O-rhamnosyl-erythronolide B

Proton	δ_H	multiplicity	coupling
2-H	2.84	dq	10.4, 6.8
3-H	3.71	d	10.4
4-H	2.16	dd	7.4, 3.6
5-H	3.52	d	3.6
7-H_a	1.94	dd	14.7, 10.3
7-H_b	1.43	dd	14.7, 2.6
8-H	2.70	m	
10-H	3.03	qd	6.9, 1.7
11-H	3.96	dd	9.7, 1.5
12-H	1.65	qd	9.7, 7.1
13-H	5.44	ddd	9.7, 4.4, 0.6
14-H_a	1.71	m	
14-H_b	1.49	m	
15-H_3	0.88	dd	7.4, 7.4
16-H_3	1.20	d	6.8
17-H_3	1.02	d	7.4
18-H_3	1.30	s	
19-H_3	1.13	d	7.1
20-H_3	0.96	d	6.8
21-H_3	0.94	d	7.1
1'-H	4.88	d	1.9
2'-H	3.94	dd	3.2, 1.9
3'-H	3.64	dd	9.5, 3.2
4'-H	3.42	dd	9.5, 9.5
5'-H	3.85	dq	9.5, 6.2
6'-H_3	1.28	d	6.2

Table 2: ^{13}C NMR Data for 3-O-rhamnosyl-erythronolide B

Carbon	δ_c
C1	175.4
C2	44.3
C3	87.5
C4	36.1
C5	80.6
C6	74.4
C7	36.3
C8	44.7
C9	219.9
C10	39.3
C11	69.5
C12	39.8
C13	74.5
C14	25.5
C15	9.3
C16	14.5
C17	7.6
C18	16.4
C19	17.4
C20	8.1
C21	8.1
C1'	103.0
C2'	70.8
C3'	70.7
C4'	72.2
C5'	69.3
C6'	16.5

Table 3: ^{1}H NMR Data for 3-O-rhamnosyl-6-deoxyerythronolide B

Proton	δ_H	multiplicity	coupling
2-H	2.90	m	
3-H	3.69	m	
4-H	1.66	m	
5-H	3.52	d	8.7
6-H	1.63	m	
7-H_a	1.89	m	
7-H_b	0.96	m	
8-H	2.68	m	
10-H	2.89	m	
11-H	3.75	dd	10.0, 1.8
12-H	1.69	m	
13-H	5.25	dd	9.1, 4.5
14-H_a	1.78	m	
14-H_b	1.53	m	
15-H_3	0.92	dd	9.4, 9.4
16-H_3	1.97	d	6.8
17-H_3	1.07	d	6.9
18-H_3	1.17	d	6.8
19-H_3	1.11	d	6.6
20-H_3	1.24	d	6.9
21-H_3	0.91	d	7.0
1'-H	4.79	d	1.6
2'-H	3.97	dd	3.2, 1.8
3'-H	3.61	dd	9.6, 3.2
4'-H	3.42	dd	9.6, 9.6
5'-H	3.69	dq	9.6, 6.2
6'H_3	1.28	d	6.2

Table 4: ^{13}C NMR Data for 3-O-rhamnosyl-6-deoxyerythronolide B

Carbon	δ_c
C1	177.0
C2	42.6
C3	83.1
C4	41.5
C5	76.4
C6	36.2
C7	33.6
C8	44.4
C9	213.3
C10	44.9
C11	70.0
C12	40.9
C13	76.0
C14	25.2
C15	5.9
C16	13.8
C17	8.5
C18	19.5
C19	14.8
C20	14.1
C21	8.3
C1'	103.0
C2'	70.6
C3'	70.8
C4'	72.2
C5'	69.4
C6'	16.6

Construction of expression plasmid for spnI,

The gene *spnI* was amplified by PCR using the primers SpnI1 5'-CTTCATATGAGTGAGATCGCAGTTGCCCCCTGGTCG -3' and SpnI2 5'-AACAGATCTGCCGCCCTCGACGCCGAGCGCTTGCC -3', which introduce a *Nde*I site at the 5' end and a *Bgl*II site at 3' end of *spnI*. Chromosomal DNA of *Saccharopolyspora spinosa* was used as a template. After treatment with T4 polynucleotide kinase the PCR product was cloned into *Sma*I-cut pUC18 and the resulting plasmid was used to transform *E. coli* DH10B. The sequence of *spnI* in the isolated plasmid was checked, and this plasmid was then digested using the restriction enzymes *Nde*I and *Bgl*II. A 1.2 kb fragment was isolated and ligated into the identically-digested vector fragment of pSG142, which introduced a C-terminal His_6-tag into SpnI. This final construct was named pSGSpnI (Fig. 6). Differences to the published DNA sequence accession AY007564 were detected (Fig. 6B).

Construction of expression plasmid for spnK

For expression of *spnK*, primers SpnK1 5'-TCATCCATATGTCCACAACGCACGAGATCGAAACCGT-3' and SpnK2 5'-TCTGCAGATCTCTCGTCCTCCGCGCTGTTCACGTCGGCCA- 3' were used to amplify *spnK* with chromosomal DNA of *Saccharopolyspora spinosa* as a template. The 1.2 kb DNA fragment was isolated, treated with T4 polynucleotide kinase and cloned into *Sma*I cut pUC18. After transformation into *E. coli* DH10B the construct was isolated and the sequence of *spnK* was verified. After digestion with *NdeI/BglII*, a 1.2 kb fragment was isolated, ligated with the vector fragment of *NdeI/BglII* digested pSG142 and used to transform *E. coli* DH10B. Plasmid pSGSpnK was isolated (Fig. 6). A C-terminal His_6-tag was introduced into SpnK.

Construction of expression plasmid for spnH

For expression of *spnH*, the primers SpnH1 5'-TTCTAGAGATCTACCACAACCTGGTATTCGTGGAGAA -3' and SpnH2 5'-AACATATGCCCTCCCAGAACGCGCTGTACCTGG -3' were used to amplify *spnH* using chromosomal DNA of *Saccharopolyspora spinosa* as a template. The

PCR fragment was isolated, treated with T4 polynucleotide kinase and cloned into *Sma*I cut pUC18. After transformation into *E. coli* DH10B the construct was isolated and the sequence of *spnH* was verified. After digestion with *NdeI/BglII*, a 0.9 kb fragment was isolated, ligated with the vector fragment of *NdeI/BglII* digested pSG142 and used to transform *E. coli* DH10B. Plasmid pSGSpnH was isolated (Fig. 6).

Isolation of the bioconversion strains SGT2pSGSpnI, SGT2pSGSpnk and SGT2pSGSpnH

Saccharopolyspora erythraea SGT2 (Gaisser *et al.*, 2000) was transformed with the plasmid constructs pSGSpnI, pSGSpnK and pSGSpnH. The transformants were verified by isolating chromosomal DNA followed by PCR analysis . The PCR products were assessed by restriction digests and the pattern of DNA fragments for *spnI*, *spnK* and *spnH* was as expected (Fig. 7, 8 and 9).

Preparation of 3-O-(2'-O-methylrhamnosyl)erythronolide B

Feeding experiments using 3-O-rhamnosyl-erythronolide B (Gaisser *et al.*, 2000) were carried out as described (Gaisser *et al.*, 1997). The cultures of the strains SGT2 and SGT2pSGSpnI, were fed with 3-O-rhamnosyl- erythronolide B, incubated at 30 °C for 3 to 5 days and analysed using electrospray mass spectrometry (Fig. 10). A novel peak was visible in supernatants of SGT2pSGSpnI with a retention time of 17.5 minutes and m/z 545 ([M- H_2O]H^+) and m/z 585 ([M]Na^+).

Isolation of 3-O-(2'-O-methylrhamnosyl)erythronolide B

1 l of DMpSGOleG2 culture supernatant containing 3-O-rhamnosyl- erythronolide B was filter sterilised and fed to cultures of SGT2pSGSpnI using standard microbiological techniques as described above. The new compound with the retention time of 17.5 minutes and m/z 545 ([M- H_2O]H^+) and m/z 585 ([M]Na^+) was isolated from the supernatant of these cultures as previously described (Gaisser et al., 2000). The novel compound was characterised as 3-O-(2'-O-methylrhamnosyl)erythronolide B (Fig. 11).

Table 5: ^{1}H and ^{13}C NMR data for 3-O-(2'-O-methylrhamnosyl)erythronolide B

Position	δ_H	Multiplicity	Coupling	δ_C
1				176.8
2	2.85	dq	10.2, 7.0	45.9
3	3.75	d	10.6	88.9
4	2.15	m		37.8
5	3.51	d	3.9	81.8
6				75.8
7	1.93	dd	14.5, 10.2	37.9
	1.43	dd	14.5, 2.7	
8	2.71	m		46.2
9				220.8
10	3.04	m		40.9
11	3.96	dd	10.4, 1.6	71.0
12	1.65	m		41.3
13	5.44	dd	9.8, 4.7	76.1
14	1.73	m		27.1
	1.49	m		
15	0.88	dd	7.4, 7.4	10.8
16	1.21	d	7.0	16.1
17	1.01	d	7.4	9.1
18	1.33	s		26.6
19	1.13	d	7.0	19.0
20	0.96	d	6.7	9.6
21	0.94	d	7.0	9.7
1'	4.93	d	1.4	100.8
2'	3.54	dd	3.3, 1.7	82.3
3'	3.68	dd	9.4, 3.2	72.1
4'	3.34	dd	9.4, 9.4	73.9
5'	3.82	dq	9.4, 6.3	70.7
6'	1.25	d	6.3	17.9
7'	3.44	s		59.1

Feeding of 3-O-(2'-O-methylrhamnosyl)erythronolide B

3-O-rhamnosyl- erythronolide B was fed to cultures of SGT2pSGSpnI followed by an incubation at 30°C. The supernatants containing 3-O-(2'-O-methylrhamnosyl)erythronolide B were centrifuged, filter sterilised and added to cultures of the strains SGT2 and SGT2pSGSpnK using standard microbiological techniques. After incubation at 30°C for several days the supernatants were analysed by electrospray mass spectrometry (Fig. 12). A new peak with a retention time of 20.7 minutes and m/z of 559 ($[M- H_2O]H^+$) and m/z of 599 ($[M]Na^+$) was detected which indicates the presence of 3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B in the culture supernatant of the strain SGT2pSGSpnK. To prepare sufficient amounts of this novel compound for NMR analysis, plasmid pSGSpnIKH was isolated.

Construction of expression plasmid pSGSpnIKH

An expression plasmid which contains both genes, *spnH* and *spnK* ,was isolated after digesting plasmid pSGSpnH with *Bgl*II and isolating the vector DNA. Plasmid pSGSpnK was digested with *Afl*II / *Nhe*I and the 1.5 kb DNA band was isolated. Fill-in reactions were performed using the isolated DNA fragments as described in Sambrook *et al.*, 1989 followed by ligation and transformation of *E. coli* DH10B. Plasmid pSGSpnHK was isolated (Fig. 13). Plasmid pSGSpnHK was digested with *Xba*I and the vector DNA was isolated. Plasmid pSGSpnI was digested with *Afl*II / *Nhe*I and the 1.5 kb DNA band was isolated. Fill-in reactions were performed using the isolated DNA fragments as described in Sambrook *et al.*, 1989 followed by ligation and transformation of *E. coli* DH10B. Plasmid pSGSpnIKH was isolated (Fig. 13) and *S. erythraea* SGT2 was transformed.

Preparation of 3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B

1 l of DMpSGOleG2 culture supernatant containing 3-O-rhamnosyl- erythronolide B was filter sterilised and fed to cultures of SGT2pSGSpnIKH using standard microbiological techniques. The new compound was isolated from the supernatant of these cultures and analysed by NMR using the methods described for the preparation

of 3-O-(2'-O-methylrhamnosyl)erythronolide B. The novel compound was characterised as 3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B (Fig. 14).

Table 6: ^{1}H and ^{13}C NMR data for 3-O-(2', 3'-bis-O-methylrhamnosyl)erythronolide B

Position	δ_H	Multiplicity	Coupling	δ_C
1				175.3
2	2.87	dq	10.2, 6.8	44.4
3	3.37	br. d	10.2	87.5
4	2.15	qdd	7.3, 3.9, 0.9	36.3
5	3.51	d	3.8	80.4
6				74.4
7	1.93	dd	14.8, 10.4	36.4
	1.44	dd	14.7, 2.7	
8	2.71	dqd	13.1, 7.1, 2.8	44.7
9				219.3
10	3.04	qd	6.8, 1.7	39.4
11	3.97	dd	10.2, 1.7	69.6
12	1.65	dqd	10.4, 7.2, 0.9	39.8
13	5.44	ddd	9.8, 4.7, 0.9	74.7
14	1.72	ddq	14.0, 9.6, 7.3	25.6
	1.50	dqd	14.0, 7.5, 4.6	
15	0.88	dd	7.4, 7.4	9.4
16	1.23	d	6.9	14.6
17	1.03	d	7.4	7.7
18	1.34	s		25.1
19	1.14	d	7.1	17.5
20	0.96	d	6.8	8.2
21	0.94	d	7.1	8.2
1'	4.95	d	1.7	99.5
2'	3.75	dd	3.2, 1.9	76.8
3'	3.37	dd	9.4, 3.0	80.5
4'	3.43	dd	9.4, 9.4	71.3
5'	3.85	dq	9.4, 6.4	69.3
6'	1.26	d	6.2	16.5
7'	3.45	s		57.6
8'	3.48	s		56.5

A small peak with m/z 613 was detected and the MS/MS analysis indicated that this peak represented 3-O-(2',3',4'-tris-O-methylrhamnosyl)erythronolide B (Fig. 15).

Formation of 3-O-rhamnosylerythromycins and 3-O-rhamnosyl-6-deoxyerythromycins

The plasmid pSGCIII was transformed into *S. erythraea* SGT2 cells to produce mutant strain SGT2, pSGCIII and culture broths of the transformed strain were analysed as described by Gaisser et al. (1997) and by Gaisser et al. (1988). Supernatants from culture broths of *S. erythraea* mutant DM (pSGOLEG2) containing 3-O-rhamnosyl-erythronolide B were fed to SGT2pSGCIII cells. Analysis of the supernants using electrospray mass spectrometry, revealed the presence of a new peak at m/z 706 corresponding to 3-O-rhamnosyl-erythromycin D.

Both compounds were isolated using a gene cassette approach as described below.

Isolation of 3-O-(2'-O-methylrhamnosyl)erythromycin D

1 l of DMpSGOleG2 culture supernatant which contained 3-O-rhamnosyl-erythronolide B was filter sterilised and fed to cultures of SGT2pSGSpnI using standard microbiological techniques. The culture supernatant was analysed for 3-O-(2'-O-methylrhamnosyl)erythronolide B and extracted as described in Materials and Methods. The crude extract was dissolved in 1 ml methanol and added to cultures of SGT2pSGeryCIII followed by an incubation at 30°C for four days. The supernatant was analysed and a major peak at m/z 720 was detected. The novel compound was analysed using the same methods as described for the preparation of 3-O-(2'-O-methylrhamnosyl)erythronolide B. The novel compound was identified as 3-O-(2'-O-methylrhamnosyl)erythromycin D (Fig. 16B).

Table 7: ^{1}H and ^{13}C NMR data for 3-O-(2′-O-methylrhamnosyl)erythromycin D

Position	δ_H	Multiplicity	Coupling	δ_C
1				175.9
2	2.93	dq	9.4, 7.3	44.6
3	4.19	d	9.0	82.1
4	2.16	m		39.4
5	3.59	overlaps with 2′		83.7
6				73.8
7	1.99	dd	14.9, 8.5	37.7
	1.54	dd	14.9, 4.7	
8	2.80	m		42.8
9				218.7
10	2.98	qd	6.8, 1.7	40.4
11	3.99	dd	10.2, 1.3	69.2
12	1.65	dq	9.8, 7.3	40.1
13	5.35	ddd	9.4, 4.7, 0.9	74.8
14	1.74	ddq	14.1, 9.4, 7.3	25.2
	1.51	dqd	14.1, 7.3, 4.7	
15	0.89	dd	7.3, 7.3	9.2
16	1.25	d	6.8	14.8
17	1.12	d	7.3	8.3
18	1.42	s		26.1
19	1.12	d	6.8	8.3
20	0.97	d	6.8	7.6
21	0.93	d	7.3	8.2
1′	5.01	d	1.3	98.0
2′	3.59	overlaps with 5		80.6
3′	3.71	dd	9.4, 3.4	70.6
4′	3.40	dd	9.4, 9.4	72.1
5′	3.75	dq	9.4, 6.0	69.8
6′	1.32	d	6.4	17.3
7′	3.46	s		57.6
1″	4.38	d	7.3	103.0
2″	3.27	dd	10.7, 7.3	70.7
3″	2.80	m		63.9
4″	1.77	m		30.2
	1.28	m		
5″	3.75	dq	9.0, 6.0	67.7
6″	1.20	d	6.0	20.2
7″	2.43	s		38.9

Bioactivity of 3-O-(2'-O-methylrhamnosyl)erythromycin D

Bacillus subtilis ATCC 6633 was used in bioassays as described previously (Gaisser et al., 1998). To assess the bioactivity of 3-O-(2'-O-methylrhamnosyl)erythromycin D, 1.1 mg aliquots of erythromycin A (Sigma) and of 3-O-(2'-O-methylrhamnosyl)erythromycin D were each dissolved in 200 µl of methanol, and series of 10-fold dilutions were prepared. Filter discs were soaked with 10 µl of these solutions and placed on 2 x TY plates overlaid with agar inoculated with an overnight culture of *B. subtilis* as described previously (Gaisser *et al.*, 1997). The development of zones of inhibition in the *B. subtilis* lawn was assessed. The size of the halos in the bacterial lawn around the filter discs indicated that the bioactivity of 3-O-(2'-O-methylrhamnosyl)erythromycin D was about 100-fold less compared to erythromycin A with *Bacillus subtilis* ATCC 6633 as indicator strain (Fig. 17).

Isolation of 3-O-(2',3'-bis-O-methylrhamnosyl)erythromycin D

1 l of DMpSGOleg2 culture supernatant which contained 3-O-rhamnosyl-erythronolide B was filter sterilised and fed to cultures of SGT2pSGSpnHKI using standard microbiological techniques. The culture supernatant was analysed for 3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B and extracted as described in Materials and Methods. The fraction which contained 3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B was isolated. The dried extract of 3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B was dissolved in 1 ml methanol and added to cultures of SGT2pSGeryCIII followed by an incubation at 30°C for four days. The supernatant was analysed. A major peak with m/z of 734 was detected. The compound was isolated and analysed using the methods described for the preparation of 3-O-(2'-O-methylrhamnosyl)erythronolide B. The novel compound was identified as 3-O-(2',3'-bis-O-methylrhamnosyl)erythromycin D (Fig. 16C).

Table 8: ^{1}H and ^{13}C NMR data for 3-O-(2′, 3′-bis-O-methylrhamnosyl)erythromycin D

Position	δ_H	Multiplicity	Coupling	δ_C
1				176.0
2	2.93	dq	9.4, 6.8	44.8
3	4.20	dd	9.4, 0.9	82.5
4	2.18	dq	7.7, 7.3	39.2
5	3.61	d	7.7	84.0
6				74.2
7	1.99	dd	14.9, 8.5	37.8
	1.52	dd	14.9, 4.7	
8	2.81	m		42.8
9				219.2
10	2.96	qd	6.8, 1.7	40.5
11	3.99	dd	10.2, 1.7	69.4
12	1.65	dqd	10.2, 7.3, 0.9	39.9
13	5.36	ddd	9.4, 4.7, 1.3	74.9
14	1.74	ddq	14.1, 9.4, 7.3	25.4
	1.50	dqd	14.1, 7.3, 4.7	
15	0.90	dd	7.3, 7.3	9.3
16	1.27	d	7.3	14.8
17	1.13	d	7.3	8.4
18	1.43	s		26.3
19	1.11	d	6.8	17.2
20	0.97	d	6.8	7.7
21	0.92	d	7.3	8.3
1′	5.02	d	2.1	98.4
2′	3.80	dd	2.6, 2.6	76.6
3′	3.39	dd	9.0, 3.0	80.4
4′	3.49	dd	9.0, 9.0	70.9
5′	3.75	dq	9.0, 6.4	70.2
6′	1.32	d	6.4	17.2
7′	3.49	s		57.5
8′	3.46	s		56.3
1″	4.44	d	7.3	102.5
2″	3.37	dd	7.3, 3.4	69.9
3″	3.21	m		65.0
4″	1.91	m		29.9
	1.42	m		
5″	3.75	m		70.0
6″	1.25	d	6.4	20.1
7″	2.68	s		38.5

Construction of the Saccharopolyspora erythraea strain SGT 3 (*ΔeryCIIIΔeryBVΔeryBVI*)

To prevent contamination with mycarosyl-erythronolide B in feeding assays, the *Saccharopolyspora erythaea* strain SGT3 (*ΔeryCIIIΔeryBVΔeryBVI*) was isolated using plasmid pHhol (Gaisser et al., 1997). The transformation of the *S. erythraea* strain DM and the isolation of the mutant SGT3 were performed as described (Gaisser et al., 1997). To investigate the thiostrepton sensitive mutants no 31, 33, 34 and 25, chromosomal DNA was analysed using PCR analysis. Chromosomal DNA was subjected to PCR using the primers as described earlier (Gaisser et al., 1997). The expected 360 bp fragment was amplified from wild type DNA and two bands of roughly 100 and 300 bp of size were detected after *Pst*I restriction digest. In samples with the chromosomal DNA of SGT3 as a template, a 300 bp fragment was amplified which was found to be resistant to digestion by *Pst*I. This result indicates the introduction of a 60 bp deletion in *eryBVI* into the genome of SGT3. This strain is used as background for the expression of the gene cassettes described below.

Construction of expression plasmid for oleP

For expression of *oleP*, the primers OleP1 5'-CTCCAGCAAAGGACACACCCATATGACCGATACGCACA -3' and OleP2 5'-CGGCAGATCTGCCGGCCGTCACCAGGAGACGATCTGG-3' were used to amplify *oleP* using plasmid 3gh2 as a template. The PCR fragment was isolated, treated with T4 polynucleotide kinase and cloned into *Sma*I cut pUC18. After transformation into *E. coli* DH10B the construct was isolated and the sequence of *oleP* was verified. After digestion with *NdeI*/*Bgl*II, a 1.3 kb fragment was isolated, ligated with the vector fragment of *NdeI*/*Bgl*II digested pSG142 and used to transform *E. coli* DH10B. Plasmid pSGOleP was isolated.

Feeding of erythronolides to strain SGT2pSGOleP

Saccharopolyspora erythraea SGT2 (Gaisser *et al.*, 2000) was transformed with the plasmid construct pSGOleP as described in Materials and Methods. The transformants were verified by isolating chromosomal DNA followed by PCR analysis. Feeding

experiments using 6-deoxyerythronolide B, erythronolide B, 3-O-mycarosyl-erythronolide B, 3-O-rhamnosyl-erythronolide B (Gaisser *et al.*, 2000), and erythromycin A were carried out as described (Gaisser *et al.*, 1997). The cultures of the strains SGT2 and SGT2pSGOleP were fed with these compounds, incubated at 30 °C for 3 to 5 days and analysed using electrospray mass spectrometry. Novel peaks were visible in supernatants of SGT2pSGOleP fed with 6-deoxyerythronolide B (m/z 434 $[M]NH_4^+$), 3-O-mycarosyl-erythronolide B (m/z 578 $[M]NH_4^+$ and m/z 580 $[M]NH_4^+$) and 3-O-rhamnosyl-erythronolide B (m/z 580 $[M]NH_4^+$ and m/z 582 $[M]NH_4^+$). Novel peaks could not be detected in supernatants that contained erythronolide B and erythromycin A. MS/MS analysis of these new compounds indicated the presence of 8,8a-epoxy- or 8,8a-dihydroxy derivatives of 6-deoxy-erythronolide B, 3-O-mycarosyl-erythronolide B, and 3-O-rhamnosyl-erythronolide B.

Preparation of 8a-hydroxy-3-O-mycarosyl erythronolide B and 8-epoxy-3-O-mycarosyl erythronolide B

2.5 l of culture supernatant of SGT2pSGOleP fed with 60 mg of 3-O-mycarosyl-erythronolide B were grown and the novel compounds were isolated using methods described in Materials and Methods. The structures of these compounds were confirmed by NMR analysis using the methods described for the preparation of 3-O-(2'-O-methylrhamnosyl)erythronolide B. The 3-O-mycarosyl erythronolide B derived compounds were identified as 8a-hydroxy-3-O-mycarosyl erythronolide B and 8,8a-epoxy-3-O-mycarosyl erythronolide B (Fig 21).

Table 9: ^1H NMR Data for 8a-hydroxy-3-O-mycarosyl erythronolide B

Proton	δ_H	multiplicity	coupling (Hz)
2-H	2.87	dq	10.1, 7.0
3-H	3.75	dd	10.0, 1.2
4-H	2.15	m	
5-H	3.55	d	3.5
7-H_a	1.49	dd	14.6, 3.0
7-H_b	1.84	dd	14.6, 10.3
8-H	2.83	m	
8a-H_a	3.66	dd	11.0, 6.0
8a-H_b	3.71	dd	11.0, 9.1
10-H	3.07	qd	6.9, 1.8
11-H	3.93	dd	10.2, 1.8
12-H	1.66	m	
13-H	5.44	ddd	9.5, 4.9, 1.2
14-H_a	1.49	dqd	16.9, 7.3, 4.8
14-H_b	1.73	ddq	16.9, 9.5, 7.3
15-H_3	0.88	d	7.3
16-H_3	1.18	d	6.9
17-H_3	1.02	d	7.3
18-H_3	1.33	s	
19-H_3	0.96	d	6.8
20-H_3	0.94	d	7.1
1'-H	5.02	dd	3.9, 1.1
2'-H_a	1.84	dd	14.5, 4.2
2'-H_b	2.08	dd	14.5, 1.2
4'-H	2.98	d	9.7
5'-H	4.06	dq	9.8, 6.2
6'-H_3	1.28	d	6.2
7'-H_3	1.22	s	

Table 10: ^{13}C NMR Data for 8a-hydroxy-3-O-mycarosyl erythronolide B

Carbon	δ_C
1	175.3
2	44.4
3	86.4
4	36.3
5	80.1
6	74.4
7	31.0
8	52.9
8a	63.5
9	217.3
10	40.6
11	69.6
12	39.6
13	74.6
14	25.4
15	9.3
16	14.4
17	7.5
18	24.7
19	7.2
20	8.2
1'	99.2
2'	40.8
3'	69.5
4'	76.3
5'	65.5
6'	16.8
7'	24.7

Table 11: ^{1}H NMR Data for 8,8a-epoxy-3-O-mycarosyl erythronolide B

Proton	δ_H	multiplicity	coupling (Hz)
2-H	2.89	dq	10.4, 7.0
3-H	3.74	dd	10.4, 1.3
4-H	2.21	m	
5-H	3.48	d	3.4
7-H_a	1.50	d	14.9
7-H_b	2.62	d	14.9
8a-H_a	2.52	d	5.5
8a-H_b	2.67	d	5.5
10-H	3.12	qd	6.8, 1.9
11-H	4.22	dd	10.4, 1.9
12-H	1.67	qd	7.0, 1.3
13-H	5.47	ddd	9.8, 4.7, 1.3
14-H_a	1.51	m	
14-H_b	1.75	m	
15-H_3	0.88	dd	7.3, 7.3
16-H_3	1.20	d	7.3
17-H_3	1.03	d	7.3
18-H_3	1.43	s	
19-H_3	0.97	d	6.8
20-H_3	0.95	d	7.0
1'-H	5.02	dd	4.2, 1.3
2'-H_a	1.84	dd	14.5, 4.3
2'-H_b	2.08	dd	14.5, 1.3
4'-H	2.98	d	9.8
5'-H	4.06	dq	9.8, 6.2
6'-H_3	1.27	d	6.2
7'-H_3	1.22	s	

Table 12: ^{13}C NMR Data for 8,8a-epoxy-3-O-mycarosyl erythronolide B

Carbon	δ_C
1	175.4
2	44.4
3	86.7
4	36.2
5	81.0
6	75.2
7	35.0
8	62.5
8a	50.0
9	210.5
10	44.1
11	68.9
12	39.5
13	74.6
14	25.5
15	9.4
16	14.6
17	7.5
18	25.9
19	7.7
20	8.2
1'	99.4
2'	40.7
3'	69.5
4'	76.2
5'	65.3
6'	16.8
7'	24.6

Preparation of 8,8a-dehydro-6-deoxyerythronolide B and 8-hydroxy-6-deoxyerythronolide B

Plasmid pSGOleP was used to transform the 6-deoxyerythronolide B producer strain *S. erythraea* SGT1 (Δ*eryBV*, Δ*eryCIII*, Δ*eryF*) as described in Materials and Methods. The transformants were verified by isolating chromosomal DNA followed by PCR analysis. Cultures of SGT1 and SGT1pSGOleP were grown as described previously (Gaisser et al., 2000) and the supernatants were analysed using electrospray mass spectrometry as described in Materials and Methods. Two major compounds in the supernatant were purified and analysed using NMR techniques as described for the preparation of 3-O-(2'-O-methylrhamnosyl)erythronolide B. The product with m/z 401 $[M-H_2O]H^+$ was identified as the 8,8a-dihydroxy derivative of 6-deoxyerythronolide B recently disclosed (Shah et al., 2000). The compound with m/z of 385 $[M]H^+$ was confirmed as 8,8a-dehydro-6-deoxyerythronolide B (Fig 22). The structure of a further, minor compound of the culture supernatant was identified as 8-hydroxy-6-deoxyerythronolide B (Fig 22).

Table 13: ^{1}H NMR Data for 8,8a-dehydro-6-deoxyerythronolide B

Proton	δ_H	multiplicity	coupling (Hz)
2-H	2.70	dq	9.8, 6.8
3-H	3.55	ovrlp	
4-H	1.69	ovrlp	
5-H	3.55	ovrlp	
6-H	1.91	m	
7-H_a	2.11	dd	17.1, 7.7
7-H_b	2.45	dd	17.1, 3.8
8a-H_a	5.38	s	
8a-H_b	5.66	s	
10-H	3.23	dq	6.8, 1.7
11-H	3.60	dd	10.2, 1.7
12-H	1.70	ovrlp	
13-H	5.24	ddd	9.4, 4.7, 2.1
14-H_a	1.52	ddq	14.1, 9.4, 7.3
14-H_b	1.77	dqd	14.1, 7.3, 4.7
15-H_3	0.90	dd	7.3, 7.3
16-H_3	1.18	d	6.8
17-H_3	1.03	d	6.8
18-H_3	1.12	d	6.8
19-H_3	0.97	d	6.8
20-H_3	0.96	d	7.3

Table 13: ^{13}C NMR Data for 8,8a-dehydro-6-deoxyerythronolide B

Carbon	δ_C
1	178.7
2	45.3
3	75.5
4	42.3
5	77.7
6	35.9
7	33.2
8	150.4
8a	120.6
9	208.8
10	44.9
11	71.9
12	42.1
13	76.9
14	26.7
15	10.9
16	15.2
17	8.9
18	20.2
19	6.5
20	10.0

Table 14: ^{1}H NMR Data for 8-hydroxy-6-deoxyerythronolide B ($CDCl_3$)

Proton	δ_H	multiplicity	coupling (Hz)
2-H	2.63	dq	10.2, 6.8
3-H	3.61	dd	10.2, 3.0
4-H	1.56	m	
5-H	3.53	br. s	
6-H	1.40	m	
7-H_a	1.74	m	
7-H_b	1.96	dd	14.9, 3.4
10-H	3.05	qd	6.8, 0.9
11-H	3.45	d	9.8
12-H	1.72	m	
13-H	5.45	ddd	9.4, 4.7, 0.9
14-H_a	1.50	m	
14-H_b	1.72	m	
15-H_3	0.90	t	7.3, 7.3
16-H_3	1.25	d	6.8
17-H_3	1.05	d	7.3
18-H_3	1.15	d	6.8
19-H_3	1.42	s	
20-H_3	1.11	d	6.8
21-H_3	0.90	d	7.3

Table 15: ^{13}C NMR Data for 8-hydroxy-6-deoxyerythronolide B ($CDCl_3$)

Carbon	δ_x
1	175.6
2	43.9
3	77.8
4	41.3
5	79.4
6	36.0
7	39.2
8	79.7
9	218.7
10	38.8
11	69.4
12	40.1
13	75.2
14	25.6
15	10.3
16	14.8
17	7.7
18	20.2
19	26.9
20	9.6
21	8.9

Strategy to isolate gene cassettes

The strategy to prepare gene cassettes with different combinations of glycosyltransferase- and methyltransferase genes is adapted from a technique previously described (WO 077181 A2) to build gene cassettes expressed under the control of the *actII*-Orf4 regulator. The expression of these gene cassettes in a suitable strain background is a powerful approach to generate novel post-PKS modified polyketides in a random or directed fashion. The method is based upon the introduction of *Xba*I restriction sites at the 3' and 5'- end of the PCR fragments. The introduction of a *Xba*I site at the 5'- end of the PCR fragment which is sensitive to the Dam methylase of the strain background will protect this site from further *Xba*I digest. To retain the Shine Dalgarno sequence 5' of the respective gene the pSG142 derived constructs which contain these genes were used as a template. Using plasmid DNA isolated from dam⁻ host strains such as *E. coli* ET12567, the amplified genes were isolated as *Xba*I fragments. Using a host strain with an active Dam methylase such as *E. coli* DM10B these fragments were sequentially cloned into gene cassettes. This technique provides the means to build gene cassettes of different length and different order using the same strategy over and over again. An overview of the strategy described here and the isolated gene cassettes is depicted in Fig. 18.

The following example of this methodology was based upon the isolation of a PCR fragment of *oleG2* into which a *Hin*dIII and a *Nde*I restriction site was introduced at the 5'-end of the fragment and a *Xba*I, *Bgl*II and *Eco*RI site at the 3'- end of the PCR fragment. This fragment was digested using the restriction enzymes *Hin*dIII and *Eco*RI and it was cloned into pUC19 which was digested identically. Plasmid pSGcas*oleG2* was isolated. The genes *spnI*, *spnK*, *spnH* and *eryCIII* were amplified using PCR techniques. A *Xba*I restriction site was introduced at the 5'- end which is sensitive to methylation by the Dam methylase of the strain background. At the 3'-end a *Xba*I site was introduced. The pSG142 derived constructs which contain these genes were used as a template. The PCR fragments were treated with T4 polynucleotide kinase as described above and cloned into *Sma*I cut pUC18. The DNA sequences of these clones were confirmed by sequencing analysis. After transforming

the constructs into a *dam*⁻ strain background, the DNA was isolated and digested using *Xba*I. The *Xba*I fragments of the inserts of around 0.8-1.3 kb of size were isolated and ligated into the *Xba*I cut pSGcasOleG2. After building the gene cassettes in pUC19, each construct was digested using the restriction enzymes *Nde*I / *Bgl*II and the DNA fragment encoding the gene cassette was isolated and cloned into the *NdeI*/ *Bgl*II digested vector DNA of pSG142. These plasmids were transformed into SGT3. The transformants were analysed as described above.

The following primers were used:

casoleG21	5' GGGGAAGCTTGCCGACGATGACGACGACCACCGGACGAACGCATCGATTAATTAAG
casoleG22	5' GGGGAATTCAGATCTGGTCTAGAGGTCAGCCCGCATGGTTCCCGCCTCCTCGTCCGC GTCCGCCGCT
casspnI3	5' GGGTCTAGATCCGGACGAACGCATCGATTAATTAAGGAGGACAGATATGAGTGAGA.. TCGCAGTTGCCCC
casspnI4	5'GGGGTCTAGAGGTCAGCCGCCCTCGACGCCGAGCGCTTGCCGGGGCACGAACCCCGG CGCGGCAGGCT
casspnK1	5'GGGTCTAGATCCGGACGAACGCATCGATTAATTAAGGAGGACAGATATGTCCACAAC GCACGAGATCG
casspnK2	5' GGGGTCTAGAGGTCACTCGTCCTCCGCGCTGTTCACGTCGGCCAGGTGCAATATGTC
caseryCIII1	5'GGGTCTAGATCCGGACGAACGCATCGATTAATTAAGGAGGACAGATATGCGCGTCGT CTTCTCCTC
caseryCIII2	5' GGGGTCTAGAGGTCATCGTGGTTCTCTCCTTCCTGCGGCCAGTTCCTCGCA

Analysis of SGT3pSGcasoleG2spnI

The clone SGT3pSGcas*oleG2spnI* was isolated using the approach described above. The cells were grown as described in Materials and Methods and the culture supernatant was analysed. As expected, 3-O-(2'-O-methylrhamnosyl)erythronolide B and 3-O-(2'-O-methylrhamnosyl)-6-deoxyerythronolide B were detected.

Analysis of SGT3pSGcasoleG2spnIspnK

SGT3pSGcas*oleG2spnIspnK* was isolated using the approach described above. The cells were grown as described in Materials and Methods and the culture supernatant was analysed. As expected, 3-O-(2'-O-methylrhamnosyl)erythronolide B and 3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B were detected (Fig. 19). The culture supernatant also contained a further novel compound which was isolated and the structure was confirmed by NMR analysis using the methods described for the preparation of 3-O-(2'-O-methylrhamnosyl)erythronolide B. The structure of the novel compound was characterised as 3-O-(2', 3'-bis-O-methylrhamnosyl)-6-deoxyerythronolide B (Fig. 19).

Table 16: ^{1}H and ^{13}C NMR data for 3-O-(2′, 3′-bis-O-methylrhamnosyl)-6-dEB

Position	δ_H	Multiplicity	Coupling	δ_C
1				178.4
2	2.89	dq	8.1, 7.3	46.2
3	3.37	overlap		84.1
4	1.68	m		43.1
5	3.49	d	9.2	77.8
6	1.66	m		37.1
7	1.01	m		35
	1.86	m		
8	2.68	m		45.8
9				218
10	2.95	qd	6.8, 1.9	43.8
11	3.73	dd	10.2, 1.7	72.1
12	1.65	m		42
13	5.25	ddd	9.2, 5.3, 1.3	77.4
14	1.54	m		26.5
	1.80	m		
15	0.92	dd	7.3, 7.3	10.8
16	1.27	d	6.4	15.3
17	1.08	d	7.0	10
18	1.16	d	6.4	20.6
19	1.13	d	6.6	16.4
20	0.98	d	6.8	7.5
21	0.92	d	7.0	9.7
1′	4.97	d	1.5	100.7
2′	3.77	overlap		78.1
3′	3.33	dd	9.4, 3.0	81.8
4′	3.42	dd	9.4, 9.4	72.8
5′	3.67	dq	9.4, 6.2	70.9
6′	1.25	d	6.0	18.1
7′	3.45	s		58.9
8′	3.47	s		57.9

Analysis of SGT3pSGoleG2spnIspnKeryCIII

SGT3pSG*oleG2spnIspnKeryCIII* was isolated using the methods described above. The cells were grown as described in Materials and Methods. The culture supernatants of SGT3pSGcas*oleG2spnIspnKeryCIII* contained only small amounts of compounds with an attached desosamine sugar residue.

Analysis of SGT3pSGoleG2spnIspnKeryCIIIhis

The PCR product using the primer combination of caseryCIII2 and SG14 was isolated using the methods described for the construction of expression plasmid for *eryCIII*. The gene cassette pSGcas*oleG2spnIspnKeryCIIIhis* was created using the approach described above. Strain SGT3pSGcas*oleG2spnIspnKeryCIIIhis* was isolated and cells were grown as described in Materials and Methods. The culture supernatant of the clone was assessed using techniques described in Materials and Methods. Compounds 3-O-mannosyl erythromycin D, 3-O-(2'-O-methyl rhamnosyl erythromycin D, 3-O-(2',3'-bis-O-methyl rhamnosyl erythromycin D), 3-O-(2'-O-methyl rhamnosyl)-6-deoxyerythromycin D and 3-O-(2',3'-bis-O-methyl rhamnosyl)-6-deoxy erythromycin D were detected (Fig.20). The introduction of the his_6-tag at the C-terminus of EryCIII therefore seems to improve glycosyl transfer of the desosamine sugar residue to its substrates. This result indicates that the expression of the last gene of the gene cassette can be improved by introducing the his_6-tag fusion at the C-terminal end of the protein.

OleP cassette

To include *oleP* into the arrangements of gene cassettes, primers OlePcass1 5'-GGGTCTAGATCCGGACGAACGCATCGATTAATTAAGGAGGACAGATATGA CCGATACGCACACCGGACCGACACC-3' and OlePcass2 5'-GGGGTCTAGAGGTCACCAGGAGACGATCTGGCGTTCCAGTCCGCGGATCA -3' were used. The PCR fragment –using plasmid pSGOleP as template - was isolated, treated with T4 polynucleotide kinase and cloned into *SmaI* cut pUC18. After transformation into *E. coli* DH10B the construct was isolated and verified. Plasmid pSGOlePcass was used to transform the *dam*⁻ *Escherichia coli* strain ET12567. The

plasmid DNA was isolated and after digestion with *XbaI*, a 1.3 kb fragment was isolated, ligated with the vector fragment of *XbaI* digested constructs and used to transform *E. coli* DH10B. An overview over these constructs is given in Fig. **23**.

Preparation of 3-O-rhamnosyl-8,8a-dehydro-6-deoxyerythronolide B

S. erythraea strain SGT1pSGcasoleG2oleP was isolated using the methods described above and 4 l of cells were grown as described in Material and Methods. The culture supernatant were isolated and analysed as described for the preparation of 3-O-(2'-O-methylrhamnosyl)erythronolide B. Two novel compounds, 3-O-rhamnosyl-8,8a-dehydro-6-deoxyerythronolide B and 3-O-rhamnosyl-8,8a-dihydroxy-6-deoxyerythronolide B were detected (Fig. 24). The structure of 3-O-rhamnosyl-8,8a-dehydro-6-deoxyerythronolide B was confirmed by NMR analysis.

Table 17: ^{1}H NMR Data for 3-O-rhamnosyl-8,8a-dehydro-6-deoxyerythronolide B

Proton	δ_H	multiplicity	coupling (Hz)
2-H	2.80	dq	7.3, 7.3
3-H	3.76	dd	7.5, 2.6
4-H	1.66	m	
5-H	3.48	dd	7.9, 2.6
6-H	2.00	m	
7-H_a	2.18	m	
7-H_b	2.39	d	16.4
8a-H_a	5.42	s	
8a-H_b	5.68	s	
10-H	3.21	m	
11-H	3.68	m	
12-H	1.72	m	
13-H	5.18	ddd	9.2, 4.9, 1.3
14-H_a	1.53	m	
14-H_b	1.77	m	
15-H_3	0.90	dd	7.3, 7.3
16-H_3	1.20	d	7.3
17-H_3	1.06	d	6.8
18-H_3	1.12	d	6.8
19-H_3	0.98	d	6.8
20-H_3	0.96	d	7.3
1'-H	4.83	obscured	
2'-H	3.96	dd	3.2, 1.7
3'-H	3.62	dd	9.6, 3.2
4'-H	3.42	dd	9.6, 9.6
5'-H	3.69	m	
6'-H_3	1.28	d	6.2

Table 18: ^{13}C NMR Data for 3-O-rhamnosyl-8,8a-dehydro-6-deoxyerythronolide B

Carbon	δ_C
1	177.3
2	45.0
3	81.1
4	42.0
5	75.5
6	33.3
7	31.2
8	148.4
8a	119.3
9	210.2
10	43.8
11	71.1
12	40.5
13	76.0
14	25.1
15	9.3
16	13.7
17	8.6
18	18.3
19	8.8
20	8.8
1'	102.4
2'	70.5
3'	70.9
4'	72.2
5'	69.3
6'	16.6

Construction of expression plasmid for oleG1

To establish which of the various possible start codons in the published sequence (accession number AJ002638) is used for the expression of *oleG1* (Fig. 25), various constructs were tested by measuring the complementation of the *eryCIII* mutation in *S. erythraea* SGT2 after feeding with 3-O-mycarosyl erythronolide B using techniques described in Materials and Methods. Complementation indicated by the production of small amounts of erythromycin A was only observed when vector pSGOleG1 was used (Fig. 26). Plasmid pSGOleG1 was isolated using the primers 7390 5'-CCGCCATATGAGCATCGCGTCGAACGGCGCGCGCTCGGC-3' Ole2 5'-TCAGATCTCCGCCTTCCCGCCATCGCGCCGGTGGCAT-3' to amplify *oleG1*. The cloning procedure was as described for the construction of the expression plasmid for oleG2. Expression vectors using the published start codon or one of the following ATG codons indicated in Fig. 25 did not complement the *eryCIII* mutation of SGT2 after feeding with 3-O-mycarosyl erythronolide B. This result indicates that the correct start codon which is required for the expression of *oleG1* is the ATG overlapping with the *oleP1* stop codon (Fig. 25).

6-deoxyerythronolide B as a substrate for oleG2, but not oleG1

The *S. erythraea* mutant SGQ1 (SGT2DeryF) was created starting with SGT2 by introducing a deletion in the *eryF* gene as described above using standard microbiological techniques. SGQ1 was transformed with the plasmid constructs pSGOleG2, pSGOleP and pSGOleG1 and feeding experiments using the sterile filtered culture supernatants of SGT1 containing 6-deoxyerythronolide B were carried out as described in Materials and Methods. The results indicate, that both, OleG2 and to a smaller extent OleP, accept 6-deoxyerythronolide B as a substrate (Fig. 27). 6-deoxyerythronolide B is not a substrate for OleG1.

3-O-rhamnosyl-6-deoxyerythronolide B as a substrate for oleP

Culture supernatants of SGT1pSGOleG2 containing 3-O-rhamnosyl-6-deoxyerythronolide B was sterile filtered and were added to cultures of SGQ1, SGQ1pSGOleG2, SGQ1pSGOleP and SGQ1pSGOleG1 using standard

microbiological techniques. The analysis of these culture supernatants indicates that 3-O-rhamnosyl-6-deoxyerythronolide B is a substrate for OleP but not for OleG1 (Fig 28).

Feeding of 8,8a-epoxy-3-O-mycarosyl erythronolide B

Cell cultures of SGT1pSGOleG1 were grown as described in Material and Methods and fed with 8,8a-epoxy-3-O-mycarosyl erythronolide B as described earlier (Gaisser et al., 2000). The culture supernatant was analysed using techniques described above and the results indicate that 8,8a-epoxy-3-O-mycarosyl erythronolide B is a substrate for OleG1.

Expression of pSGcassOleG2EryCIII in SGT3

The gene *ole*G2 was amplified using the primers casoleG21 and casoleG22 (see above) and the DNA of plasmid pSGOleG2 as a template (Gaisser *et al.*, 2000). The PCR product was ligated into *Sma*I cut pUC18 and transformed into the *Escherichia coli* strain DH10B. The sequence of the PCR product was verified. The resulting plasmid was digested using the restriction enzymes *Eco*RI and *Hind*III followed by a ligation into *Eco*RI*Hind*III digested pUC19 and transformation of *E. coli* DH10B. The resulting plasmid was named pSGcassOleG2.

The gene *eryCIII* was amplified using the primers caseryCIII and caseryCIII2 (see above) and the DNA of plasmid pSGEryCIII as a template. The PCR product was ligated into *Sma*I cut pUC18 and transformed into the *Escherichia coli* strain DH10B. The sequence of the PCR produce was verified. The resulting plasmid was transformed into the *dam Escherichia coli* strain ER12567. The DNA of the transformant was isolated and digested using the restriction enzyme *Xba*I. The 1.3 kb DNA fragment was isolated and ligated into *Xba*I digested pSGcassOleG2 and transformed into *E. coli* DH10B. The correct orientation of *eryCIII* was assessed using restriction digests and plasmid pOleG2EryCIII was isolated. A DNA band of about 2.8 kb was isolated after a restriction digest using *Nde*I, *Bg*II and *Dra*I followed by a ligation into the *Nde*I/*Bg*II digested expression vector pSG142 and

transformation of *E. coli*DH10B. Plasmid pSGcassOleG2EryCIII was isolated and used to transform the S. *erythraea* mutant SGT3. Thiostrepton resistant colonies were selected. Culture supernatants of these strains were isolated as described (Gaisser *et al.*, 1997) and analysed using electrospray mass spectrometry. A peak with the retention time of 9.2 and the m/z of 706 was detected which indicates the presence of rhamnosyl-erthyromycin D in the supernatant. Another peak with m/z 690 was also found which indicates the presence of rhamnosyl-6-deoxyerythromycin D in the supernatant (see Fig 16).

Anti-microbial activity of the resulting erythromycin analogues was demonstrated through development of zones of inhibition in a lawn of erythromycin-sensitive *Bacillus subtilis* around plugs of the transformed cells in a standard bioassay.

Isolation of 5-O-glucosyl- and 5-O-desosaminyl-tylactone

Analysis of the tylactone standard using electrospray mass spectrometry showed a major peak at m/z 377 and minor peaks at m/z 359 and m/z 417. When tylactone was supplied to strain SGT2, in which both *eryBV* and *eryCIII* are deleted, peaks at m/z 557 and 579 were detected in the culture supernatants which would correspond to glucosylated derivatives of tylactone. This confirmed the presence of another glycosyltransferase in *S. erythraea* which accepts tylactone as a substrate. However, the analysis of the culture supernatant of *S. erythraea* SGT2(pSGTYLM2) fed with tylactone revealed a major peak at m/z 552, which fragmented into peaks at m/z 158 and m/z 359 in MS/MS experiments, indicating the presence of 5-*O*-desosaminyl-tylactone in the expression medium of *S. erythraea* SGT2(pSGTYLM2). The putative glucosyl- and desosaminyl-tylactones (2.2 mg and 2.0 mg respectively from 1.5 l of culture broth) were analysed and the structures were fully confirmed as 5-*O*-glucosyl- and 5-*O*-desosaminyl-tylactone by using ^{1}H and ^{13}C NMR (see Figs 5A and 5B respectively).

Table 19: ^{1}H NMR Data for 5-desosaminyl-tylactone

Proton	δ_H	Multiplicity	Coupling
2-H_a	2.49	dd	17.4, 9.6
2-H_b	2.04	d	17.4
3-H	3.72	d	9.6
4-H	1.70	m	
5-H	3.77	d	9.6
6-H	1.11	m	
7-H_a	1.72	m	
7-H_b	1.45	m	
8-H	2.64	m	
10-H	6.47	m	15.4
11-H	7.27	d	15.4
13-H	5.70	d	10.4
14-H	2.81	ddq	10.2,10.2,6.5
15-H	4.72	ddd	9.7,9.7,2.5
16-H_a	1.90	m	
16-H_b	1.60	m	
17-H_3	0.96	dd	7.4,7.4
18-H_3	1.04	D	6.8
19-H_a	1.60	m	
19-H_b	1.42	m	
20-H_3	0.85	dd	7.2,7.2
21-H_3	1.19	d	6.9
22-H_3	1.84	br.s	
23-H_3	1.08	d	6.5
1'-H	4.28	d	7.3
2'-H	3.37	dd	10.5,7.3
3'-H	3.20	ddd	11.4,11.4,4.0
4'-H_a	1.93	m	
4'-H_b	1.44	m	
5'-H	3.61	dq	10.2,6.1
6'-H_3	1.26	d	6.1
7'$(CH_3)_2$	2.70	s	

Table 20: ^{13}C NMR Data for 5-desosaminyl-tylactone

Carbon	δ_c
C1	173.6
C2	39.7
C3	67.0
C4	40.9
C5	78.6
C6	39.4
C7	33.3
C8	44.7
C9	205.5
C10	118.2
C11	148.1
C12	133.3
C13	146.0
C14	38.3
C15	78.4
C16	23.9
C17	8.4
C18	8.3
C19	20.5
C20	10.8
C21	16.3
C22	11.4
C23	14.3
C'	103.3
C2'	69.3
C3'	65.0
C4'	29.7
C5'	67.8
C6'	19.5
N7'-$(CH_3)_2$	38.5

Table 21: ^{1}H NMR Data for 5-glucosyl-tylactone

Proton	δ_H	Multiplicity	Coupling
2-H_a	2.49	dd	17.4, 9.6
2-H_b	2.05	d	17.4
3-H	3.74	br. d	9.9
4-H	1.68	m	
5-H	3.77	br. d	9.4
6-H	1.11	m	
7-H_a	1.72	m	
7-H_b	1.48	m	
8-H	2.66	dqd	11.8, 6.9, 3.6
10-H	6.44	d	15.5
11-H	7.23	d	14.9
13-H	5.67	d	10
14-H	2.78	ddq	10.3,10.3,6.6
15-H	4.68	ddd	9.1,9.1,2.7
16-H_a	1.86	m	
16-H_b	1.58	m	
17-H_3	0.94	dd	7.3,7.3
18-H_3	1.04	d	6.9
19-H_a	1.43	m	
19-H_b	1.64	m	
20-H_3	0.87	t	7.3
21-H_3	1.19	d	6.9
22-H_3	1.85	br.s	
23-H_3	1.07	d	6.6
1'-H	4.28	d	7.8
2'-H	3.17	dd	9.2,7.8
3'-H	3.33	m	
4'-H	3.34	m	
5'-H	3.19	m	
6'-H_a	3.82	dd	11.6, 2.6
6'-H_b	3.72	dd	11.6, 4.8

Table 22: ^{13}C NMR Data for 5-glucosyl-tylactone

Carbon	δ_c
C1	173.6
C2	39.8
C3	66.9
C4	41.1
C5	79.1
C6	39.6
C7	33.4
C8	45.1
C9	205.7
C10	118.6
C11	148.2
C12	133.9
C13	146.3
C14	38.4
C15	78.6
C16	24.3
C17	8.6
C18	8.2
C19	21.3
C20	10.9
C21	16.5
C22	11.1
C23	14.9
C'	103.1
C2'	74.3
C3'	76.7
C4'	70.2
C5'	75.9
C6'	61.2

Production of 23-hydroxy 5-O-mycaminosyl tylactone

The *S. erythraea* strain SGT2pSGTylM2 was grown as described previously (Gaisser et al., 1997). Tylactone was fed to these cultures after 48h (compared to 24h in previous feedings). Analysis of the supernatants using electrospray mass spectroscopy revealed the presence of a new peak at m/z 552 which was identified as 5-O-desosaminyl-tylactone. A second peak with m/z 568 was also detected in this supernatant and MS/MS analysis of this compound confirmed the presence of 5-O-mycaminosyl-tylactone in the expression medium of *S. erythaea* strain SGT2pSGTylM2. An aliquot of this supernatant was used to feed cultures of SGT2pSGTYLH. Analysis of the supernatants revealed a shift of the 5-O-mycaminosyl-tylactone peak of m/z 568 to 584 m/z. This result indicates that 5-O-mycaminosyl-tylactone was further processed by the expression of the TylH locus (Fouces et al., 1999). The TylH locus consists of two genes, tylH1 (3Fe-4S-type ferrodoxin) and tylH2 (P450 type cytochrome), postulated to form the oxidoreduction system involved in C23 oxidation) to produce 23-hydroxy 5-O-mycaminosyl tylactone in the culture supernatant.

Production of 23-O-rhamnosyl 5-O-mycaminosyl tylactone

An aliquot of the supernatant containing 5-O-mycaminosyl-tylactone was used to feed cultures of *S. erythraea* SGT2pSGTYLHN. Analysis of the supernatants using electrospray mass spectrosopy revealed the presence of a new peak at m/z 730. The shift by m/z 146 indicated the presence of 23-rhamnosyl 5-O-mycaminosyl tylactone in the culture supernatant.

Isolation of 5-O-(2'-O)-bis-glucosyl-tylactone

Cultures of SGT2pSGOLED are fed with tylactone. The supernatants of these cultures contain a new product with m/z consistent with the structure of diglucosyl-tylactone. The compound was purified as described above and the structure fully confirmed by ^{1}H and ^{13}C NMR

Table 23: ^{1}H NMR Data for 5-O(2'-O)- bis-glucosyl-tylactone

Proton	δ_H	multiplicity	coupling
2-H_a	2.05	d	17.5
2-H_b	2.48	dd	17.5, 9.6
3-H	3.73	br. d	9.8
4-H	1.74	m	
5-H	3.78	d	9.4
6-H	1.11	m	
7-H_a	1.45	m	
7-H_b	1.75	m	
8-H	2.65	m	
10-H	6.46	d	15.3
11-H	7.23	d	15.3
13-H	5.66	d	10.2
14-H	2.78	ddq	10.2, 10.2, 6.4
15-H	4.68	ddd	10.2, 9.4, 2.6
16-H_a	1.58	m	
16-H_b	1.86	m	
17-H_3	0.94	dd	7.3, 7.3
18-H_3	1.07	d	6.8
19-H_a	1.43	m	
19-H_b	1.63	m	
20-H_3	0.88	dd	7.3, 7.3
21-H_3	1.21	d	6.8
22-H_3	1.85	s	
23-H_3	1.07	d	6.6
1'-H	4.42	d	7.7
2'-H	3.43	m	
3'-H	3.52	dd	9.0, 9.0
4'-H	3.37	m	
5'-H	3.21	m	
6'-H_a	3.70	m	
6"-H_b	3.82	dd	11.5, 2.6
1"-H	4.56	d	7.7
2"-H	3.27	m	
3"-H	3.37	m	
4"-H	3.28	m	
5"-H	3.29	m	
6"-H_a	3.71	m	
6"-H_b	3.89	br. d	11.9

Table 24: ^{13}C NMR Data for 5-O(2'-O)- bis-glucosyl-tylactone

Carbon	δ_C
C1	173.4
C2	40.0
C3	66.9
C4	40.9
C5	78.7
C6	39.6
C7	33.5
C8	45.2
C9	205.7
C10	118.8
C11	148.5
C12	133.9
C13	146.4
C14	38.5
C15	78.5
C16	24.4
C17	8.6
C18	8.3
C19	21.1
C20	11.1
C21	16.6
C22	11.8
C23	15.0
C1'	101.0
C2'	81.7
C3'	76.2
C4'	70.1
C5'	75.8
C6'	61.2
C1"	104.3
C2"	74.2
C3"	76.6
C4"	70.0
C5"	77.1
C6"	61.6

Using the approach described for the creation of gene cassettes a strategy was developed to isolate 5-O-mycaminosyl-erythromycin A and 5-O-mycaminosyl-(4''-O-mycarosyl)-erythromycin A (Fig. 32).

Isolation of a gene cassette encoding the mycaminose biosynthetic pathway

A gene cassette was isolated encoding the genes responsible for the synthesis of TDP-D-mycaminose by amplifying *tylMIII*, *tylB* and *tylMI* from the tylosin biosynthetic gene cluster (accession numbers sf08223 and x81885) using chromosomal DNA of *Streptomyces fradiae* and the following primers:

TylM31

5' GGCGGGGAGAGAGGAGAGCATATGAACACGGCAGCCGGCCCGACC

TylM32

5' CCCCCTCTAGAGGTCACTCGGGGACATACGGGGCGACGGGCAGCCG

TylMI1

5'GGGGGTCTAGATCTTAATTAAGGAGGACAACCATGGCCCATTCATCCGCCACGGCCGG ACCGCAGGCCGA

TylMI2

5'GGGGGTCTAGAGGCATATGTGTCCTCCTTAATTAATCACCGGGTTTCTCCCTTCGCTCCG GGGAGCCCGGT

TylB1

5'CCCCCTCTAGATCTTAATTAAGGAGGACACCCATGACAGGGCTGCCGCGGCCCGCCGTC CGGGTG

TylB2

5'GGGGGTCTAGAGGTCACGGGCCTTCCTCCCAGGAGTCCAGCGCGGCGGA

The PCR fragments were cloned into *SmaI* cut pUC18 using standard cloning techniques as described in Materials and Methods. The sequences of the cloned fragments were verified by DNA sequence analysis. No difference to the published sequence was detected for *tylMI* or *tylM3*, but changes were detected in *tylB* which

resulted in the change of 8 amino acid regions compared to the published sequence of TylB (Fig.33).

The gene cassettes were assembled in pUC18 using the approach described above (Fig. 34). The constructs pUC18tyl*MIII-tylB* and pUC18*tylMIII-tylB-tylMI* were isolated and confirmed by restriction digests. Plasmid pUC18*tylMIII-tylB-tylMI* was digested with *Nde*I and the insert of about 3.5 kb was isolated and ligated into *Nde*I digested pSGCIII, pSGTYLM2, pSGDESVII and pSGTYLCV (Fig.35). The correct orientation was confirmed using restriction digests.

Isolation of S. erythraea GG1

Plasmid pNCO62 (Gaisser et al., 1997) was isolated from a *dam⁻ Escherichia coli* host strain and digested with the restriction enzymes *BalI / BclI*. To introduce a 0.9 kb deletion into *eryCIV* as previously described (Salah-Bey et al., 1998) the ends of the DNA fragment were filled-in using standard microbiological techniques followed by a ligation step and electroporation of *E. coli* DH10B. Plasmid pGG17 was isolated and confirmed by sequence analysis and restriction digest. To introduce a selectable marker into this construct, a 1.1 kb fragment containing the thiostrepton resistance gene was isolated using plasmid pIB060 and ligated into pGG17 to generate pGG1 (Fig. 36). This plasmid was used to introduce the *eryCIV* deletion into the genome of *S. erythraea* wild type. To isolate the *S. erythraea* strain GG1 techniques described previously (Gaisser et al., 1998) were used.

Isolation of S. erythraea SGQ2

Plasmid pGG1 was used to introduce a 0.9 kb deletion in *eryCIV* (Salah-Bey et al., 1998) into the *S. erythraea* mutant strain SGT2 to create the quadruple mutant SGQ2, using the microbiological techniques described previously (Gaisser et al., 1998). To verify the mutant, plasmid pSGCIII was used to transform SGQ2 and SGQ2pSGCIII was isolated. The cells were grown as described in Material and Methods and feeding with 3-O-mycarosyl erythronolide B was carried out as described (Gaisser et al., 2000). The supernatant of the cell culture was assessed

using techniques described in Materials and Methods and two novel peaks with 750 m/z and 713 m/z were detected (Fig.37). Using MS/MS techniques described in Materials and Methods these novel compounds were identified as 5-O-mycaminosyl-erythromycin A and 3,5-di-O-mycarosyl-erythronolide B.

Improved production of 5-O-mycaminosyl-erythromycin A

The plasmid produced by the cloning of the mycaminose gene cassette tylMIII-tylB-tylM1 in correct orientation into the NdeI site of the plasmid pSGCIII was used to transform SGQ2 and strain SGQ2p(mycaminose)CIII was isolated. The cells were grown as described in Materials and Methods and feeding with mycarosyl-erythronolide B was carried out as described (Gaisser et al., 2000). The supernatant of the cell culture was analysed using HPLC-MS as desribed in Materials and Methods and peaks with 750 m/z and 713 m/z were detected, but the amount of the material with 750 m/z, corresponding to 5-O-mycaminosyl-erythromycin A, was significantly increased relative to the other peaks.

Construction of expression plasmid for tylCV

For expression of *tylCV* the primers TylCV1 5'-GCCTGACGAAGGGTCCTGCCATATGGCTCATATTGCATT and TylCV2 5'-GCGTGGGCCGGCCGGAGATCTGGCCGCGGGGGACAGCA were used to amplify *tylCV* using genomic DNA of *S. fradiae* as template. The PCR fragment was isolated and cloned as described for the construction of expression plasmid of *eryCIII*. After digestion with *Nde*I / *Bgl*II a 1.2 kb fragment was isolated, ligated into pSG142 digested with the same restriction enzymes and used transform *E. coli* DH10B as described above. Plasmid pSGTYLCV was isolated.

Production of 5-O-mycaminosyl-(4"-O-mycarosyl) erythromycin A

The plasmid pSGTylCV was used to transform SGQ2 and strain SGQ2pSGTylCV was isolated. The cells were grown as described in Materials and Methods and a filtered supernatant from strain SGQ2p(mycaminose)CIII , containing 5-O-mycaminosyl-erythromycin A, was carried out as described for similar experiments

previously (Gaisser et al., 2000). The supernatant of the cell culture of strain SGQ2pSGTylCV was analysed using HPLC-MS as described in Materials and Methods and a novel peak with 894 m/z was detected, corresponding to 5-O-mycaminosyl-(4"-O-mycarosyl) erythromycin A.

5

References

The references cited herein are all incorporated by reference.

Caffrey, P. *et al.*, (1992) *FEBS* **304:** 225-228.

Devereux, J. *et al.*, (1984) *Nucl Acids Res* **12:** 387-395.

Fouces, R. *et al.*, (1999) *Microbiol* **145:** 855-868.

Gaisser, S. *et al.*, (1997) *Mol Gen Genet* **256:** 239-251.

Gaisser, S. *et al.*, (2000) *Mol Microbiol* **36:** 391-401.

Gandecha, A.R *et al.*, (1997) *Gene* **184:** 197-203.

Haydock, S.F. *et al.*, (1991) *Mol Gen Genet* **230:** 120-128.

Hernandez, C. *et al.*, (1993) *Gene* **134:** 139-140.

Hessler, P.E. *et al.*, (1997) *Appl Microbiol Biotechnol* **47:** 398-404.

Kaneda, T. *et al.*, (1962) *J Biol Chem* **237:** 322-327.

Katz, E. *et al.*, (1983) *J Gen Microbiol* **129:** 2703-2714.

Pereda, A. *et al.*, (1997) *Gene* **193:** 65-71.

Sambrook , J. *et al.*, (1989) 2nd ed. Cold Spring Harbor Laboratory Press, N.Y.

Sanger, F. *et al.*, (1977) *Proc Natl Acad Sci USA* **74:** 5463-5467.

Staden, R. (1984) *Nucl Acids Res* **12:** 521-528.

Weber, J.M. *et al.*, (1985) *J Bacteriol* **164:** 425-433.

Yamamoto, H. *et al.*, (1986) *J Antibiot* **34:** 1304-1313.

Xue, Y. *et al.*, (1998) *Proc Natl Acad Sci USA* **95:** 12111-12116.

Olano *et al.*, (1998) *Mol Gen Genet* **259:** 299-308.

Rodriguez *et al.*, (1995) *FEMS Microbiol. Letters* **127**: 117-120.

Shah *et al.*, (2000) *J Antibiot* **53:** 502-508.

Spagnoli *et al.*, (1983) *J Antibiot* **36:** 365-375.

Claims:

1. A process for producing a hybrid glycosylated product by transferring one or more sugar moieties to an aglycone template, the process comprising:

transforming microorganism host cells with nucleic acid encoding a glycosyltransferase (GT); and,

providing an aglycone template to the GT so that GT transfers one or more sugar moieties to the aglycone template to produce a hybrid glycosylated product; wherein one or more of the sugar moiety or moieties, the aglycone template, the GT or the host cells are heterologous to the other components.

2. The process of claim 1, wherein the aglycone template and the sugar moiety or moieties are heterologous to each other.

3. The process of claim 1, wherein the aglycone template and/or the sugar moiety or moieties are heterologous to the host cells.

4. The process of claim 1, wherein the aglycone template, the sugar moiety or moieties and the GT are heterologous to the host cells.

5. The process of claim any one of claims 1 to 4, wherein the host cell is transformed with a gene or genes for producing the sugar moiety.

6. The process of any one of claims 1 to 5, wherein the glycosyltransferase is:

(a) from the erythromycin pathway of Saccharopolyspora erythraea, desosaminyltransferase eryCIII or mycarosyltransferase eryBV;

(b) from the megalomycin pathway of Micromonospora megalomicea, desosaminyltransferase megCIII, mycarosyltransferase megBV or megosaminyltransferase;

(c) from the oleandomycin pathway of Streptomyces antibioticus, oleandrosyltransferase oleG2 (also transfers rhamnose and olivose) or desosaminyltransferase oleG1;

(d) from the tylosin pathway of Streptomyces fradiae, mycaminosyltransferase tylMII, deoxyallose transferase tylN or mycarosyltransferase tylCV;

(e) from the midecamycin pathway of Streptomyces mycarofaciens, mycaminosyltransferase midI, deoxyallose transferase or mycarosyltransferase;

(f) from the pikromycin/narbomycin pathway of Streptomyces venezuelae, desosaminyltransferase desVII;

(g) from the spinosyn pathway of Saccharopolyspora spinosa, rhamnosyltransferase or forosaminyltransferase;

(h) from the amphotericin pathway of Streptomyces nodosus, mycaminosyltransferase amphDI;

(i) from the avermectin pathway of Streptomyces avermitilis, oleandrosyltransferase;

(j) from the nystatin pathway of Streptomyces, mycaminosyltransferase,

(k) from the polyene 67-121C pathway of Actinoplanes caerulens, mycosaminyltransferase, mannosyltransferase (transferring to the mycosamine);

(l) from the elloramycin pathway of Streptomyces olivaceaous Tü2353, rhamnosyltransferase elmGT;

(m) from the mithramycin pathway of Streptomyces argillaceus, olivosyltransferase mtmGIV;

(n) from the daunomycin pathway of Streptomyces peucetius, daunosaminyltransferase dnrS;

(o) from the urdamycin pathway of Streptomyces fradiae Tü2717, rhodinosyltransferase urdGT1c, olivosyltransferase urdGT1b, rhodinosyltransferase urdGT1a and olivosyltransferase urdGT2.

7. The process of any one of the preceding claims, further comprising employing an enzyme for modifying the sugar moiety and/or the aglycone template, either before or after attachment of the sugar moiety to the aglycone template.

8. The process of claim 7, wherein the enzyme is a methyltransferase or a P450.

9. The process of claim 7 or claim 8, wherein the host cells is transformed with a heterologous gene encoding said enzyme.

10. The process of any one of the preceding claims, further comprising transforming the host cells with a recombinant polyketide synthase gene or genes so that the aglycone template is produced by the host cells

11. The process of claim 10, wherein the recombinant PKS genes are selected from the group consisting of natural PKS genes, mutated versions of natural PKS genes, hybrid PKS genes consisting of portions from at least two different natural Type I PKS gene clusters, natural Type II PKS gene clusters and a library of hybrid PKS genes of either Type I or Type II.

13. The process of claim 12, wherein the macrolide aglycone template is 6-deoxy erythronolide B, erythronolide B or tylactone, or derivatives thereof.

14. The process of any one of the preceding claims, wherein the aglycone template is selected from the group consisting of a polyketide, a mixed polyketide-peptide and a peptide.

15. The process of any one of the preceding claims, wherein the aglycone template is a polyketide.

16. The process of claim 15, wherein the polyketide is selected from the group consisting of a Type I and Type II polyketides.

17. The process of claim 14 or claim 15, wherein the PKS gene or genes produce a macrolide aglycone template.

18. The process of any one of the preceding claims which comprises supplying one or more exogenous aglycone templates to the cells.

19. The process of any one of the preceding claims which comprises employing one or more endogenous aglycone templates.

20. The process of any one of the preceding claims, further comprises deleting or inactivating one or more genes in the microorganism host cells involved in the production of the aglycone template and/or in its subsequent processing, thereby to suppress or alter the production of the natural aglycone template or product.

21. The process of any one of claims 1 to 20, comprising the steps of:

producing one of the aglycone template or the sugar moiety in first host cells as a first product;

optionally, purifying the first product from a culture of the first host cells; and,

adding the first product to a second host cell comprising one or more genes encoding the other of the aglycone template or the sugar moiety and one or more glycosyltransferases, so that the sugar moiety is transferred to the glycosyltransferase, to produce the hybrid glycosylated product.

22. The process of claim 21, wherein the first product is a filtered supernatant from the culture of the first host cells.

23. The process of claim 21, wherein the first product is isolated from the culture of the first host cells.

24. A hybrid glycosylation product as obtainable by the process of any one of claims 1 to 23.

25. A hybrid glycosylated products which comprises:

(a) one or more natural sugars linked to an erythronolide at the 7-position; or,

(b) one or more rhamnose or substituted (e.g. methyl) rhamnose sugars linked to an erythronolide; or,

(c) one or more mycarose or substituted mycarose sugars linked to an erythronolide;

and combinations of (a), (b) and (c) sugar substituents on an erythronolide.

26. A hybrid glycosylated products which comprises:

(a) one or more natural sugars linked to erythromycin at the 7-position; or,

(b) one or more mycarose or substituted mycarose sugars linked to an erythromycin; or,

(c) one or more mycaminose or substituted mycaminose sugars linked to an erythromycin;

and combinations of (a), (b) and (c) sugar substituents on an erythromycin.

27. A hybrid glycosylated products which comprises:

(a) one or more glucose or substituted glucose sugars linked to a tylactone; or,

(b) one or more desosaminose or substituted desosaminose sugars linked to a tylactone;

(c) one or more mycaminose or substituted mycaminose sugars linked to a tylactone; or

(d) one or more rhamnose or substituted rhamnose sugars linked to a tylactone;

and combinations of (a), (b), (c) and (d) sugars substituents such as a rhamnose and a mycaminose sugar linked to a tylactone.

28. A compound which is:

3-O-(2'-O-methylrhamnosyl)erythronolide B,

3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B,

3-O-(2',3',4'-tris-O-methylrhamnosyl)erythronolide B,

3-O-mycarosyl-erythronolide B,

8a-hydroxy-3-O-mycarosyl erythronolide B,

8,8a-epoxy-3-O-mycarosyl erythronolide B,

8,8a-dehydro-6-deoxyerythronolide B,

8-hydroxy-6-deoxyerythronolide B,
3-O-(2'-O-methylrhamnosyl)erythromycin D,
3-O-(2',3'-bis-O-methylrhamnosyl)erythromycin D,
3-O-(2',3',4'-tris-O-methyl rhamnosyl)erythromycin D,
5-O-mycaminosyl-erythromycin A,
5-O-mycaminosyl-4''-O-mycarosyl erythromycin A,
5-O-glucosyl-tylactone,
5-O-desosaminyl-tylactone,
23-O-rhamnosyl 5-O-mycaminosyl tylactone,
5-O(2'-O)- bis-glucosyl-tylactone,
3-O-rhamnosyl-8,8a-dehydro-6-deoxyerythronolide B,
3-O-rhamnosyl-8,8a-dihydroxy-erythronolide B, or
3,5 di-O-mycarosyl erythronolide B.

29. A host cell transformed with nucleic acid encoding a glycosyltransferase (GT), wherein the GT is heterologous to the host cells and transfers one or more sugar moieties to an aglycone template within the cells to produce a hybrid glycosylated product.

30. The host cell of claim 29, wherein the host cell is further transformed with one or more auxiliary genes.

31. The host cell of claim 30, wherein the auxiliary gene is a sugar pathway gene encoding a protein involved in the biosynthesis of a sugar moiety, thereby enabling a host cell transformed with the expression cassette to produce sugar moieties for subsequent transfer to an aglycone template.

32. The host cell of any one of claims 29 to 31 which is a strain of actinomycete,

33. The host cell of claim 32, wherein the actinomycete strain is selected from the group consisting of *Saccharopolyspora erythraea*, *Streptomyces coelicolor*,

Streptomyces avermitilis, *Streptomyces griseofuscus*, *Streptomyces cinnamonensis*, *Streptomyces fradiae*, *Streptomyces longisporoflavus*, *Streptomyces hygroscopicus*, *Micromonospora griseorubida*, *Streptomyces lasaliensis*, *Streptomyces venezuelae*, *Streptomyces antibioticus*, *Streptomyces lividans*, *Streptomyces rimosus*, *Streptomyces albus*, *Amycolatopsis mediterranei*, and *Streptomyces tsukubaensis*.

34. A process for producing a hybrid glycosylated product, the process comprising culturing the host cell of any one of claims 29 to 33 and isolating the product thus produced.

35. A process for producing a library which comprises a plurality of hybrid glycosylated products, the process comprising:

transforming microorganism host cells with nucleic acid encoding one or more glycosyltransferases (GT); and,

providing one or more aglycone templates to the GTs so that the GTs transfer one or more sugar moieties to the aglycone templates to produce said plurality of hybrid glycosylated products;

wherein one or more of the sugar moiety or moieties, the aglycone template, the glycosyltransferase or the host cells are heterologous to the other components.

36. The process of claim 35, wherein the host cell is further transformed with one or more auxiliary genes.

37. The host cell of claim 36, wherein the auxiliary gene is a sugar pathway gene encoding a protein involved in the biosynthesis of a sugar moiety, thereby enabling a host cell transformed with the expression cassette to produce sugar moieties for subsequent transfer to an aglycone template.

38. The process of any one of claims 35 to 37, further comprising screening the library for a hybrid glycosylated product having a desired characteristic.

39. The process of any one of claims 35 to 38, wherein the library comprises at least two different hybrid glycosylated products.

40. The process of any one of claims 35 to 38, wherein the library comprises at least 10 different hybrid glycosylated products.

41. The process of any one of claims 35 to 38, wherein the library comprises at least 100 different hybrid glycosylated products.

42. The process of any one of claims 35 to 41, further comprising isolating a host cell producing a desired hybrid glycosylated product.

43. The process of claim 42, further comprising culturing the host cells and isolating the hybrid glycosylated product thus produced.

44. The process of any one of claims 35 to 43, wherein endogenous polyketide biosynthesis is suppressed.

45. The process of claim 44, wherein the cells are cultured in the presence of a suppressor of endogenous polyketide synthesis.

46. The process of claim 45, wherein endogenous polyketide biosynthesis is suppressed by mutating, deleting or inactivating one or more of the PKS genes naturally present within the cells.

47. An expression cassette comprising one or more glycosyltransferase genes and one or more auxiliary genes, operably linked under the control of a promoter.

48. The expression cassette of claim 47, wherein the auxiliary gene is a sugar pathway gene encoding a protein involved in the biosynthesis of a sugar moiety,

thereby enabling a host cell transformed with the expression cassette to produce sugar moieties for subsequent transfer to an aglycone template.

49. The expression cassette of claim 48, wherein the auxiliary gene encodes an enzyme involved in the processing of a sugar moiety or an aglycone template, either before or after the sugar moiety is transferred to the aglycone by the glycosyltransferase.

50. The expression cassette of claim 49, wherein the enzyme is a methyltransferase or a P450 enzyme.

51. The expression cassette of any one of claims 47 to 50, wherein the genes are linked in a contiguous head to tail assembly.

52. The expression cassette of any one of claims 47 to51, wherein the gene or genes are introduced into the cassette by:

introducing *Xba*I restriction sites at the 3' and 5'- ends of a PCR fragment comprising the gene or genes; and

cloning the *Xba*I flanked fragment into a host strain with an active Dam methylase.

53. The expression cassette of any one of claims 47 to 52, wherein the genes are under the control of a single promoter.

54. The expression cassette of claim 53, wherein the promoter is a strong promoter.

55. The expression cassette of claim 54, wherein the genes are under the control of the *actII*-Orf4 regulator.

56. The expression cassette of any one of claims 47 to 55, wherein the cassette comprises a nucleic acid sequence encoding a histidine tag adjacent the terminal gene in the expression cassette.

57. A process of producing an expression cassette of any one of claims 47 to 56, the process comprising operably linking the genes together under the control of a promoter.

58. The process of claim 57, further comprising transforming a host cell with the expression cassette and expressing the genes comprised within it to produce the GT and proteins encoded by the auxiliary genes.

59. A host cell produced by the process of claim 58.

Fig.1A.

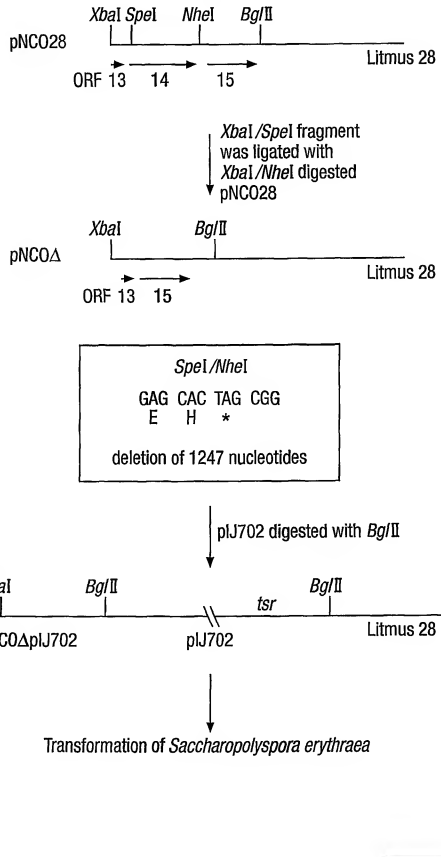

XbaI SpeI NheI BglII
pNCO28
Litmus 28
ORF 13 14 15
XbaI/SpeI fragment
was ligated with
XbaI/NheI digested
pNCO28
XbaI BglII
pNCOΔ
Litmus 28
ORF 13 15
SpeI/NheI
GAG CAC TAG CGG
E H *
deletion of 1247 nucleotides
pIJ702 digested with BglII
XbaI BglII BglII
tsr
pNCOΔpIJ702
pIJ702
Litmus 28
Transformation of Saccharopolyspora erythraea

Fig.1B.

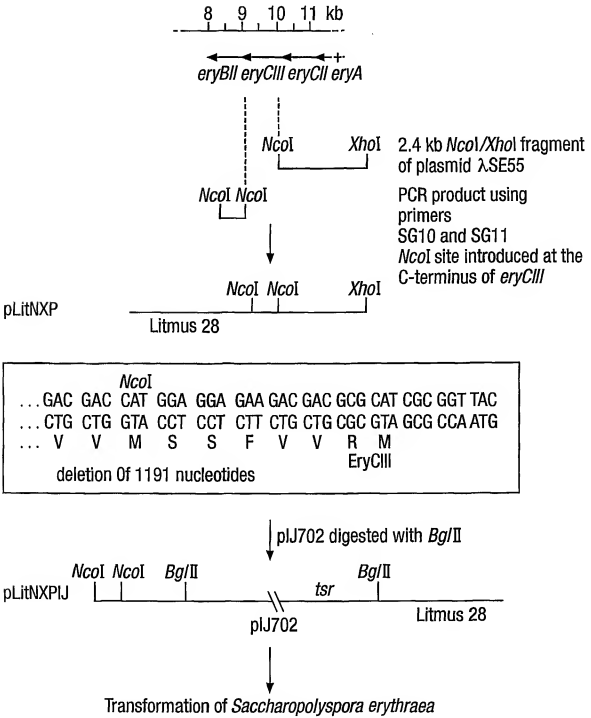

8 9 10 11 kb
eryBII eryCIII eryCII eryA
NcoI XhoI
2.4 kb NcoI/XhoI fragment of plasmid λSE55
NcoI NcoI
PCR product using primers SG10 and SG11 NcoI site introduced at the C-terminus of eryCIII
NcoI NcoI XhoI
pLitNXP
Litmus 28
NcoI
...GAC GAC CAT GGA GGA GAA GAC GAC GCG CAT CGC GGT TAC
...CTG CTG GTA CCT CCT CTT CTG CTG CGC GTA GCG CCA ATG
... V V M S S F V V R M
EryCIII
deletion Of 1191 nucleotides
pIJ702 digested with BglII
NcoI NcoI BglII
BglII
pLitNXPIJ
tsr
Litmus 28
pIJ702
Transformation of Saccharopolyspora erythraea

Fig.2.

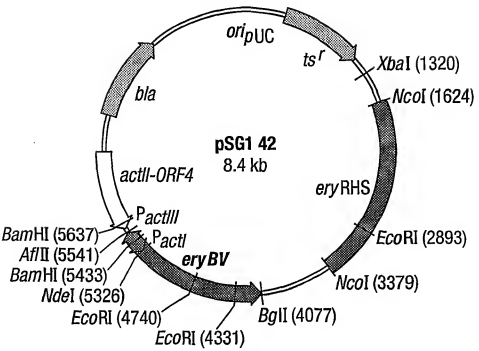
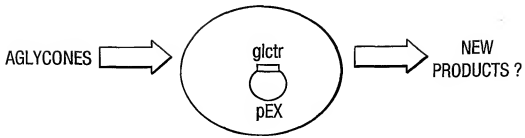

ori pUC
ts r
XbaI (1320)
NcoI (1624)
bla
pSG1 42
8.4 kb
actII-ORF4
eryRHS
PactIII
PactI
BamHI (5637)
AflII (5541)
BamHI (5433)
NdeI (5326)
EcoRI (4740)
eryBV
EcoRI (4331)
BglII (4077)
NcoI (3379)
EcoRI (2893)

Fig.3.



ΔeryBVΔeryCIIIΔeryA

Fig.3.

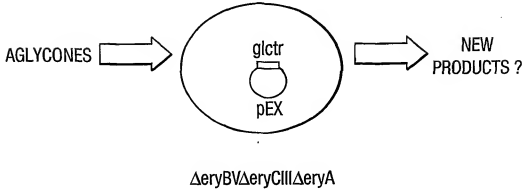
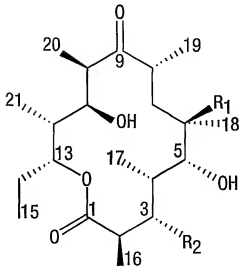


Fig.4.



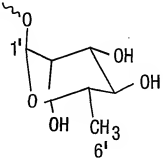
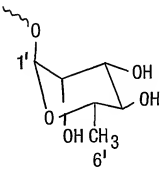
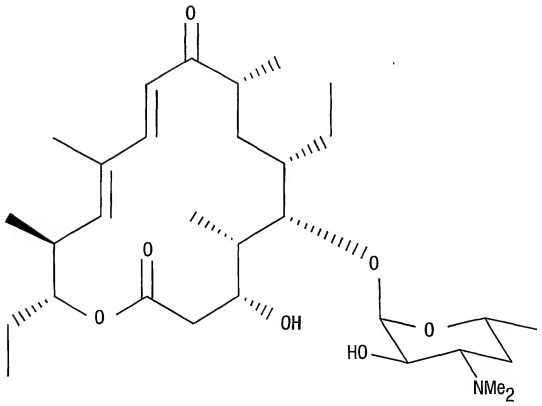
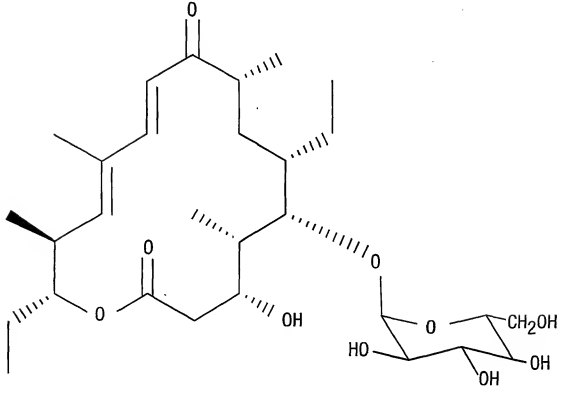
R_1=OH	R_2=OH	Erythronolide B
R_1=H	R_2=OH	6-Deoxyerythronolide B
R_1=OH	R_2 	3-O-Rhamnosyl-erythronolide B
R_1=H	R_2 	3-O-Rhamnosyl-6-deoxyerythronolide B

Fig.5A.



5-O-Desosaminyl-tylactone

Fig.5B.



5-O-Glucosyl-tylactone

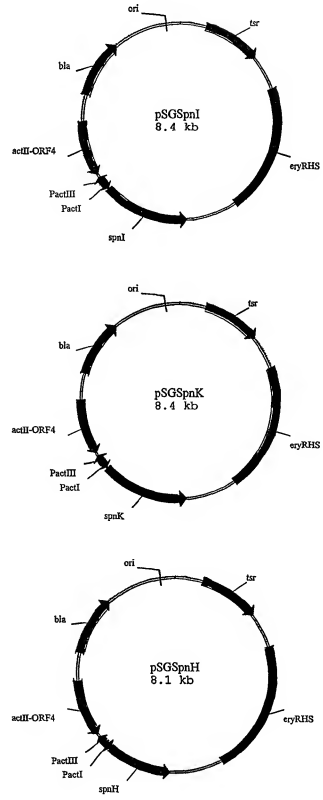

ori
tsr
bla
pSGSpnI
8.4 kb
actII-ORF4
eryRHS
PactIII
PactI
spnI
ori
tsr
bla
pSGSpnK
8.4 kb
actII-ORF4
eryRHS
PactIII
PactI
spnK
ori
tsr
bla
pSGSpnH
8.1 kb
actII-ORF4
eryRHS
PactIII
PactI
spnH

Fig. 6

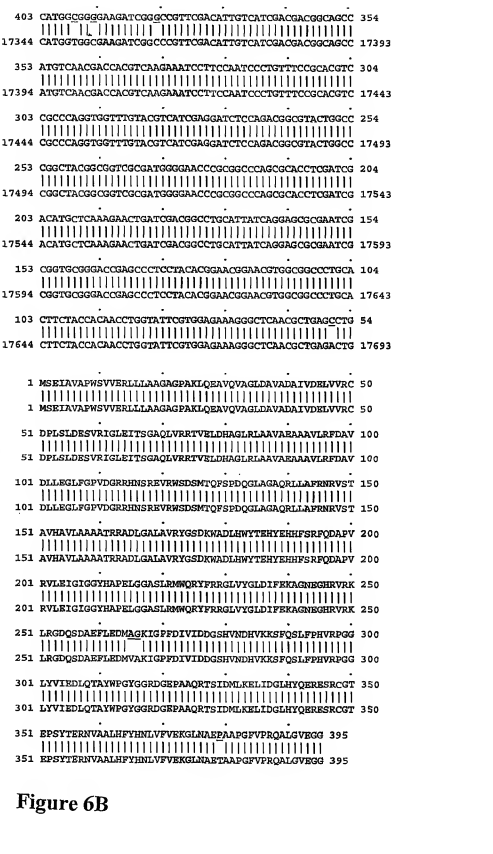
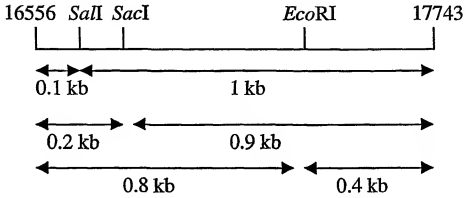
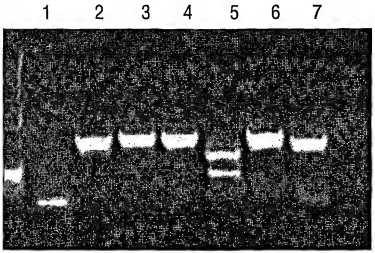

403 CATGGCGGGGAAGATCGGGCCGTTCGACATTGTCATCGACGACGGCAGCC 354
17344 CATGGTGGCGAAGATCGGCCCGTTCGACATTGTCATCGACGACGGCAGCC 17393
353 ATGTCAACGACCACGTCAAGAAATCCTTCCAATCCCTGTTTCCGCACGTC 304
17394 ATGTCAACGACCACGTCAAGAAATCCTTCCAATCCCTGTTTCCGCACGTC 17443
303 CGCCCAGGTGGTTTGTACGTCATCGAGGATCTCCAGACGGCGTACTGGCC 254
17444 CGCCCAGGTGGTTTGTACGTCATCGAGGATCTCCAGACGGCGTACTGGCC 17493
253 CGGCTACGGCGGTCGCGATGGGGAACCCGCGGCCCAGCGCACCTCGATCG 204
17494 CGGCTACGGCGGTCGCGATGGGGAACCCGCGGCCCAGCGCACCTCGATCG 17543
203 ACATGCTCAAAGAACTGATCGACGGCCTGCATTATCAGGAGCGCGAATCG 154
17544 ACATGCTCAAAGAACTGATCGACGGCCTGCATTATCAGGAGCGCGAATCG 17593
153 CGGTGCGGGACCGAGCCCTCCTACACGGAACGGAACGTGGCGGCCCTGCA 104
17594 CGGTGCGGGACCGAGCCCTCCTACACGGAACGGAACGTGGCGGCCCTGCA 17643
103 CTTCTACCACAACCTGGTATTCGTGGAGAAAGGGCTCAACGCTGAGCCTG 54
17644 CTTCTACCACAACCTGGTATTCGTGGAGAAAGGGCTCAACGCTGAGACTG 17693
1 MSEIAVAFWSVVERLLLAAGAGPAKLQEAVQVAGLDAVADAIVDELVVRC 50
1 MSEIAVAFWSVVERLLLAAGAGPAKLQEAVQVAGLDAVADAIVDELVVRC 50
51 DPLSLDESVRIGLEITSGAQLVRRTVELDHAGLRLAAVAEAAAVLREFDAV 100
51 DPLSLDESVRIGLEITSGAQLVRRTVELDHAGLRLAAVAEAAAVLREFDAV 100
101 DLLEGLPGPVDGRRHNSREVRWSDSMTQPSPDQGLAGAQRLLAFRNRVST 150
101 DLLEGLPGPVDGRRHNSREVRWSDSMTQPSPDQGLAGAQRLLAFRNRVST 150
151 AVHAVLAAAATTRADLGALAVRYGSDKWADLHWYTEHYEHHFSRFQDAPV 200
151 AVHAVLAAAATTRADLGALAVRYGSDKWADLHWYTEHYEHHFSRFQDAPV 200
201 RVLEIGIGGYHAPELGGASLRMWQRYFRRGLVYGLDIFEKAGNEGHRVRK 250
201 RVLEIGIGGYHAPELGGASLRMWQRYFRRGLVYGLDIFEKAGNEGHRVRK 250
251 LRGDQSDAEFLEDMAGKIGFFDIVIDDGSHVNDHVKKSFQSLFPHVRDGG 300
251 LRGDQSDAEFLEDMVAKIGFFDIVIDDGSHVNDHVCKSFQSLFPHVRPGG 300
301 LYVIEDLQTAYWPGYGGRDGEPAAQRTSIDMLKELIDGLHYQERESRCGT 350
301 LYVIEDLQTAYWPGYGGRDGEPAAQRTSIDMLKELIDGLHYQERESRCGT 350
351 EPSYTERNVAALHFYHNLVFVEKGLNAEPAAPGFVPRQALGVEGG 395
351 EPSYTERNVAALHFYHNLVFVEKGLNAETAAPGFVPRQALGVEGG 395

Figure 6B

Fig.7.

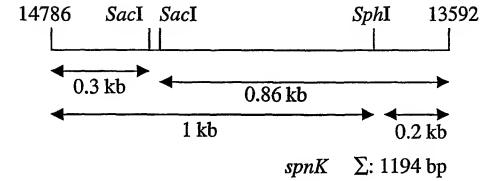


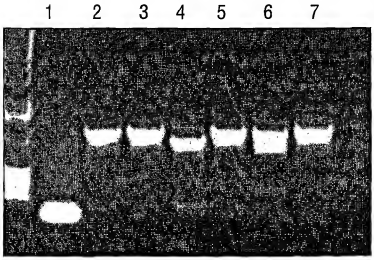
spnI: Σ: 1187 bp



1: control SGT2 genomic DNA and primers: no PCR product
2: SGT2pSGSpnI: undigested
3: SGT2pSGSpnI: *Sma*I digested
4: SGT2pSGSpnI: *Sph*I digested
5: SGT2pSGSpnI: *Eco*RI digested
6: SGT2pSGSpnI: *Sac*I digested
7: SGT2pSGSpnI: *Sal*I digested

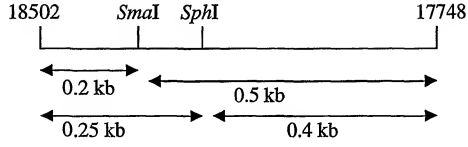
Fig.8.



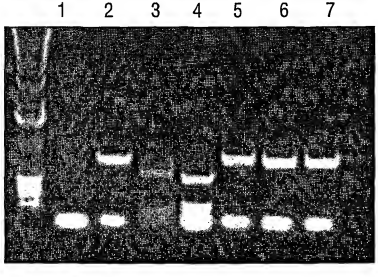


1: control SGT2 genomic DNA and primers: no PCR product
2: SGT2pSGSpnK: undigested
3: SGT2pSGSpnK: *SmaI* digested
4: SGT2pSGSpnK: *SphI* digested
5: SGT2pSGSpnK: *EcoRI* digested
6: SGT2pSGSpnK: *SacI* digested
7: SGT2pSGSpnK: *SalI* digested

Fig.9.



spnH Σ: 754 bp



1: control SGT2 genomic DNA and primers: no PCR product
2: SGT2pSGSpnH: undigested
3: SGT2pSGSpnH: *Sma*I digested
4: SGT2pSGSpnH: *Sph*I digested
5: SGT2pSGSpnH: *EcoR*I digested
6: SGT2pSGSpnH: *Sac*I digested
7: SGT2pSGSpnH: *Sal*I digested

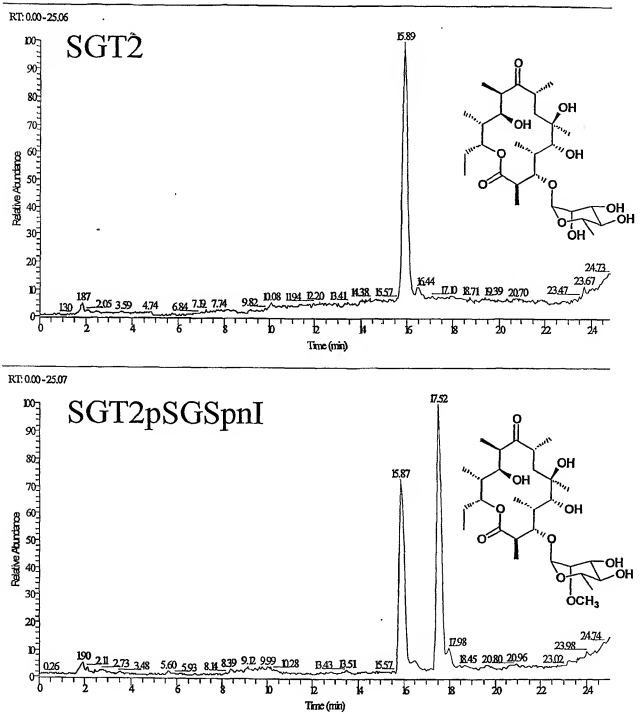
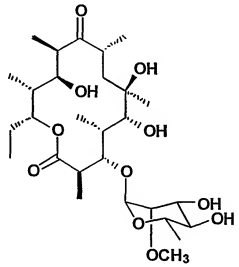

RT: 0.00 - 25.06
SGT2
Relative Abundance
Time (min)
15.89
RT: 0.00 - 25.07
SGT2pSGSpnI
17.52
15.87
OCH3

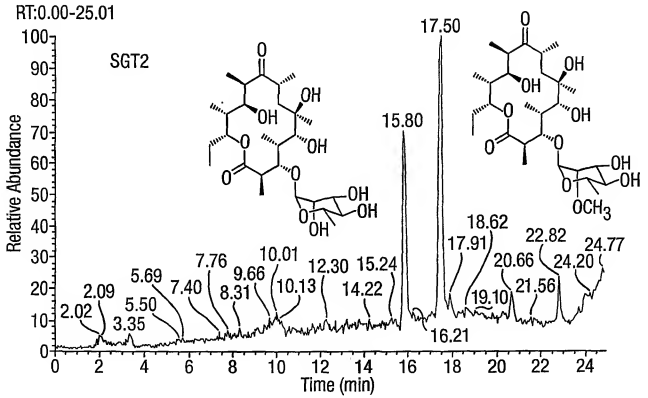
Fig. 10

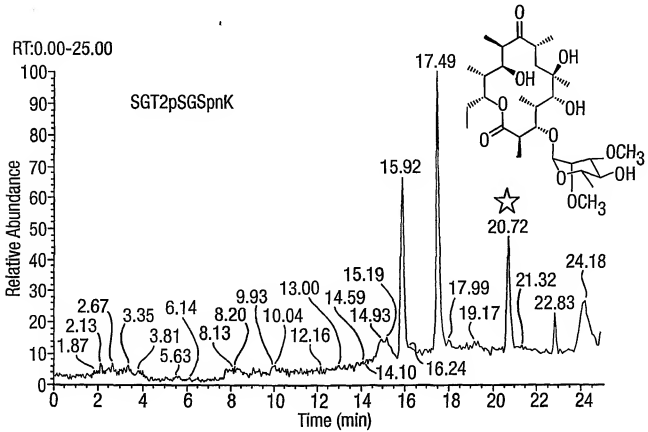


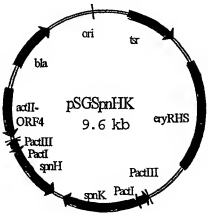
3-O-(2'-O-methylrhamnosyl)erythronolide B

Fig. 11

Fig.12.


RT:0.00-25.01
SGT2
Relative Abundance
Time (min)
17.50
15.80
16.21
18.62
17.91
22.82
24.77
20.66
24.20
21.56
19.10
10.01
7.76
9.66
12.30
15.24
5.69
7.40
8.31
10.13
14.22
2.09
5.50
2.02
3.35
OH
OCH3


RT:0.00-25.00
SGT2pSGSpnK
Relative Abundance
Time (min)
17.49
15.92
20.72
24.18
21.32
22.83
17.99
19.17
15.19
13.00
14.59
14.93
9.93
10.04
8.20
6.14
12.16
8.13
3.35
3.81
2.67
2.13
1.87
5.63
14.10
16.24
OH
OCH3


ori
tsr
bla
actII-
ORF4
pSGSpnHK
9.6 kb
eryRHS
PactIII
PactI
spnH
PactIII
spnK
PactI

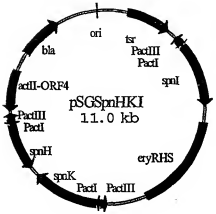
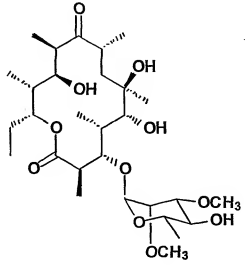
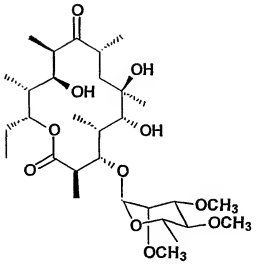

ori
tsr
PactIII
PactI
bla
spnI
actII-ORF4
pSGSpnHKI
11.0 kb
PactIII
PactI
spnH
eryRHS
spnK
PactI
PactIII

Fig. 13



3-O-(2',3'-bis-O-methylrhamnosyl)erythronolide B

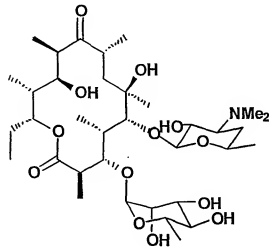
Fig. 14



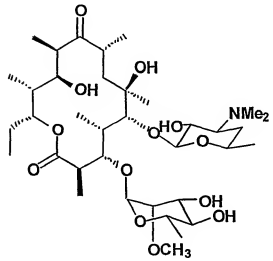
3-O-(2',3',4'-tris-O-methylrhamnosyl)erythronolide B

Fig. 15

A


O
OH
OH
NMe2
HO
O
O
O
O
O
OH
OH
O
OH

B


O
OH
OH
NMe2
HO
O
O
O
O
O
OH
OH
O
OCH3

C

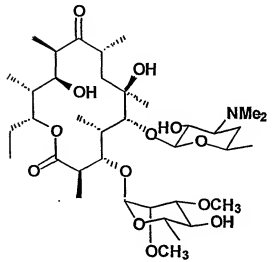
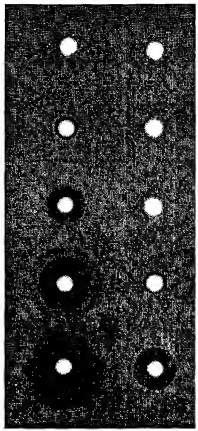

O
OH
OH
NMe2
HO
O
O
O
O
O
OCH3
OH
O
OCH3

Fig. 16

Fig.17.


Erythromycin A
3-O-(2'-O-Methylrhamnosyl) Erythromycin D
10^{-1}
10^{-2}
10^{-3}
10^{-4}

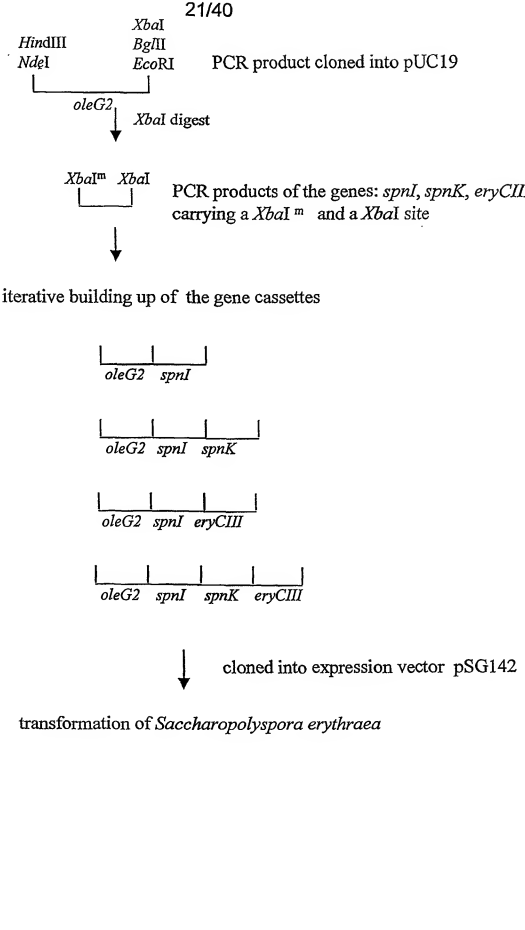

XbaI
HindIII
BglII
NdeI
EcoRI
PCR product cloned into pUC19
oleG2
XbaI digest
XbaI^m XbaI
PCR products of the genes: spnI, spnK, eryCIII carrying a XbaI m and a XbaI site
iterative building up of the gene cassettes
oleG2 spnI
oleG2 spnI spnK
oleG2 spnI eryCIII
oleG2 spnI spnK eryCIII
cloned into expression vector pSG142
transformation of Saccharopolyspora erythraea

Fig. 18

Fig.19.

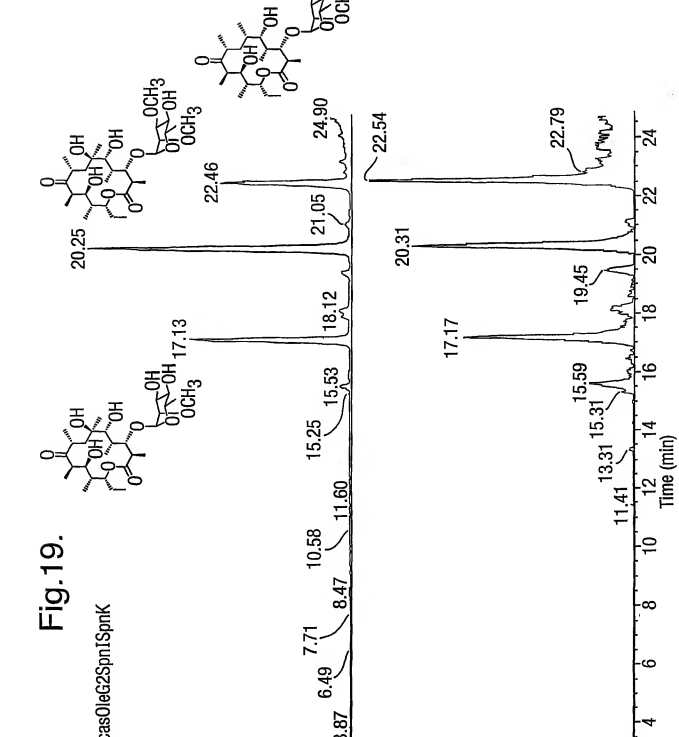
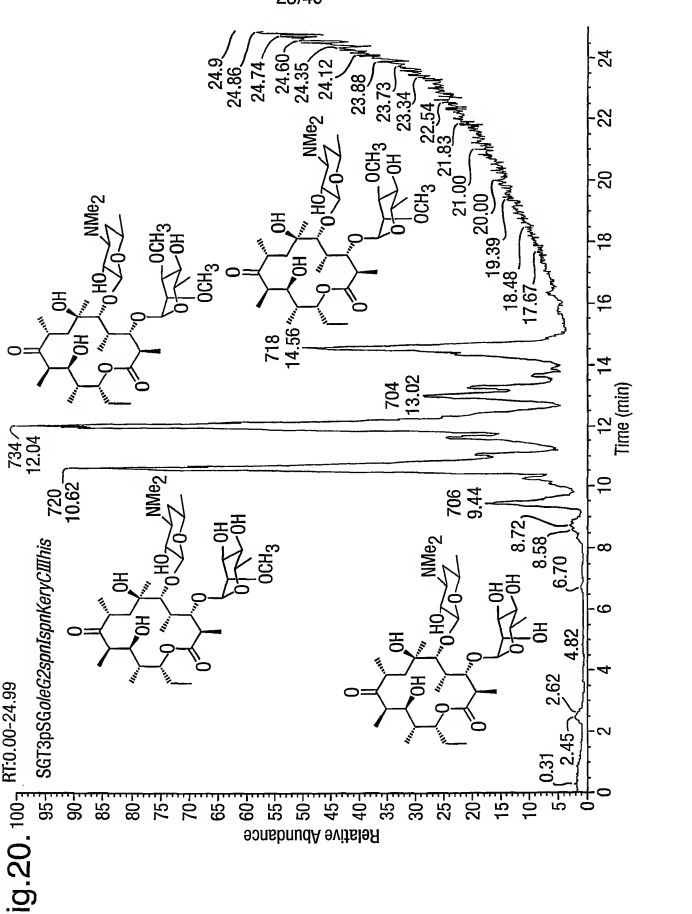
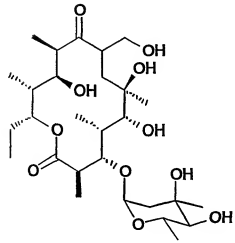
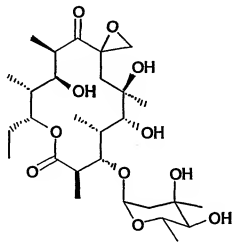

t:0.00-24.99
SGT3pSGeasOleG2SpnISpnK
Relative Abundance
Time (min)
0.67
2.66
3.87
6.49
7.71
8.47
10.58
11.60
11.41
13.31
15.31
15.59
15.25
15.53
17.17
17.13
18.12
19.45
20.31
20.25
21.05
22.46
22.54
22.79
24.90
OH
OCH3

Fig.20.


RT:0.00-24.99
SGT3pSGoleG2spnIspnkerylCIIIhis
Relative Abundance
Time (min)
734
12.04
720
10.62
718
14.56
704
13.02
706
9.44
0.31
2.45
2.62
4.82
6.70
8.58
8.72
17.67
18.48
19.39
20.00
21.00
21.83
22.54
23.34
23.73
23.88
24.12
24.35
24.60
24.74
24.86
24.9

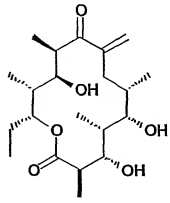


8a-hydroxy-3-O-mycarosyl erythronolide B

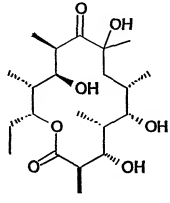


8,8a-epoxy-3-O-mycarosyl erythronolide B

Fig. 21



8,8a-dehydro-6-deoxyerythronolide B



8-hydroxy-6-deoxyerythronolide B

Fig. 22

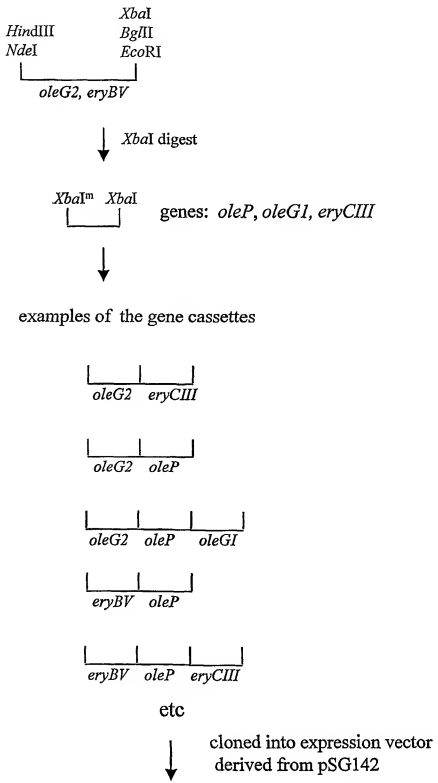
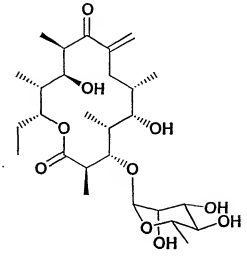
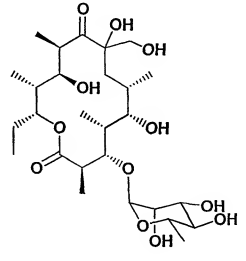

XbaI
HindIII
BglII
NdeI
EcoRI
oleG2, eryBV
XbaI digest
XbaIm XbaI
genes: oleP, oleG1, eryCIII
examples of the gene cassettes
oleG2 eryCIII
oleG2 oleP
oleG2 oleP oleGI
eryBV oleP
eryBV oleP eryCIII
etc
cloned into expression vector
derived from pSG142
transformation of Saccharopolyspora erythraea

Fig. 23



3-O-rhamnosyl-8,8a-dehydro-6-deoxyerythronolide B



3-O-rhamnosyl- 8,8a-dihydroxy-6-deoxyerythronolide B

Fig. 24

```
          CCCCGGCTGACGGCGGCGGGACCCGTCGTACGACGGCGGCGTTCCCCTGTCGTCGGCGGG
     1300 +---------+---------+---------+---------+---------+---------+ 1359
          GGGGCCGACTGCCGCCGCCCTGGGCAGCATGCTGCCGCCGCAAGGGGACAGCAGCCGCCC

a         P  R  L  T  A  A  G  P  V  V  R  R  R  R  S  P  V  V  G  G  -

          CYTOCHROME P450 MONOOXYGENASE OLEP1

          CTGCACCGGGCTCCGGTGGCCGCCGCATGAGCATCGCGTCGAACGGCGCGCGCTCGGCCC
     1360 +---------+---------+---------+---------+---------+---------+ 1419
          GACGTGGCCCGAGGCCACCGGCGGCGTACTCGTAGCGCAGCTTGCCGCGCGCGAGCCGGG

a         L  H  R  A  P  V  A  A  A  *
c           A  P  G  S  G  G  R  R  M  S  I  A  S  N  G  A  R  S  A  P  -

          CCCGCCGGCCCCTGCGCGTGATGATGACCACCTTCGCGGCCAACACGCACTTCCAGCCGC
     1420 +---------+---------+---------+---------+---------+---------+ 1479
          GGGCGGCCGGGGACGCGCACTACTACTGGTGGAAGCGCCGGTTGTGCGTGAAGGTCGGCG

c           R  R  P  L  R  V  M  M  T  T  F  A  A  N  T  H  F  Q  P  L  -

                          OLEG1 START (PUBLISHED)

          TGGTTCCCCTGGCCTGGGCAC
     1480 +---------+---------+ 1500
          ACCAAGGGGACCGGACCCGTG

c           V  P  L  A  W  A   -
```

Fig. 25

Fig.26.

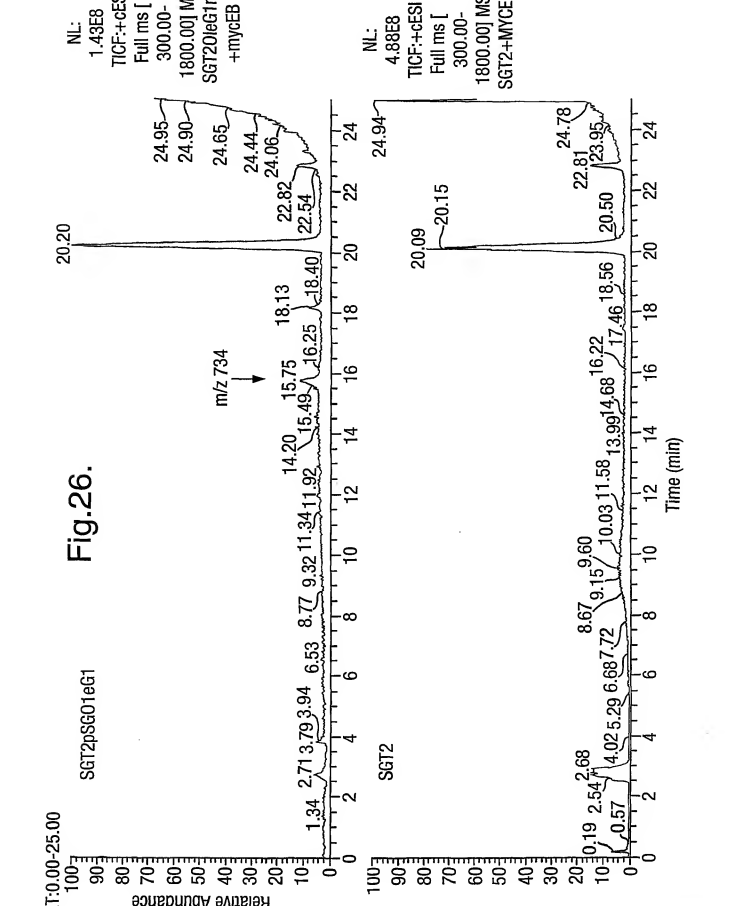
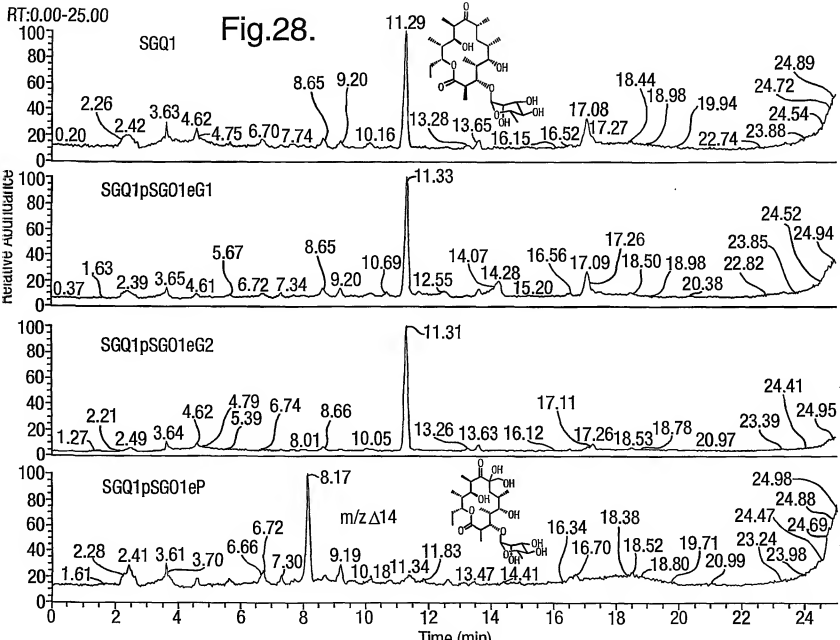
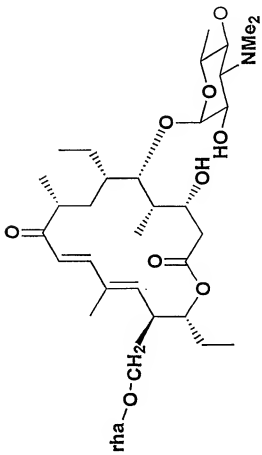

RT:0.00-25.00
Relative Abundance
SGT2pSGO1eG1
SGT2
m/z 734
NL:
1.43E8
TICF:+cESI
Full ms [
300.00-
1800.00] MS
SGT2OleG1no1
+mycEB
NL:
4.88E8
TICF:+cESI
Full ms [
300.00-
1800.00] MS
SGT2+MYCEB
Time (min)

Fig.27.

RT:0.00-25.00
SGQ1
SGQ1pSGQ1eG1
SGQ1pSGQ1eG2
SGQ1pSGQ1eP
Relative Abundance
Time (min)

Fig.28.

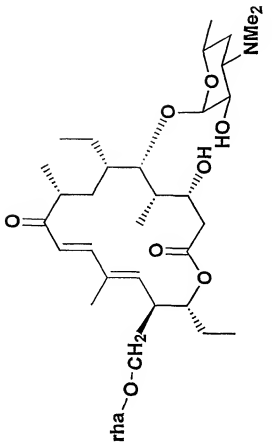

RT:0.00-25.00
SGQ1
NL:4.09E7
TIC F:+cESI Full
ms [
100.00-1500.00]
MS
SGQ1+
SN_SGT1OleG2
SGQ1pSGO1eG1
NL:7.36E7
TIC F:+cESI Full
ms [
100.00-1500.00]
MS
SGQ1pSGO1eG1n
o1+
SN_SGT1OleG2
SGQ1pSGO1eG2
NL:1.81E8
TIC F: +cESIFull
ms [
100.00-1500.00]
MS
SGQ1pSGO1eG2n
o1+
SN_SGT1OleG2
SGQ1pSGO1eP
m/z Δ14
NL:3.44E7
TIC F:+cESI Full
ms[
1100.00-1500.00]
MS
SGQ1pSGOlePno1
+SN_SGT1OleG2
Relative Abundance
Time (min)



23-O-rhamnosyl-5-O-mycaminosyl-tylactone

rha = rhamnose

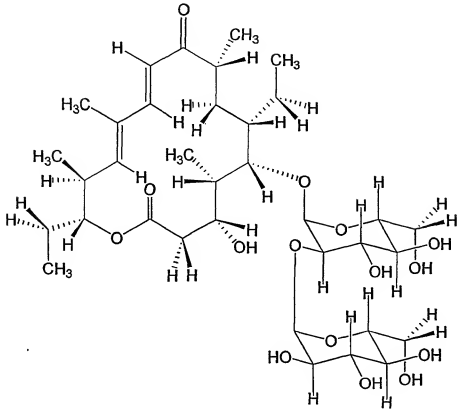
Figure 29



23-O-rhamnosyl-5-O-desosaminyl-tylactone

rha = rhamnose

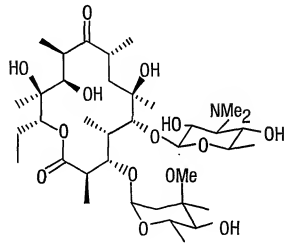
Figure 30



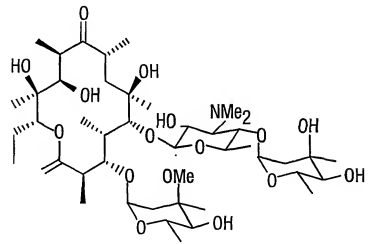
5-O-(2'-O-)-bis-glucosyl-tylactone

Fig.31

Fig.32.



3-O-mycarosyl-5-O-mycaminosyl-erythromycin A

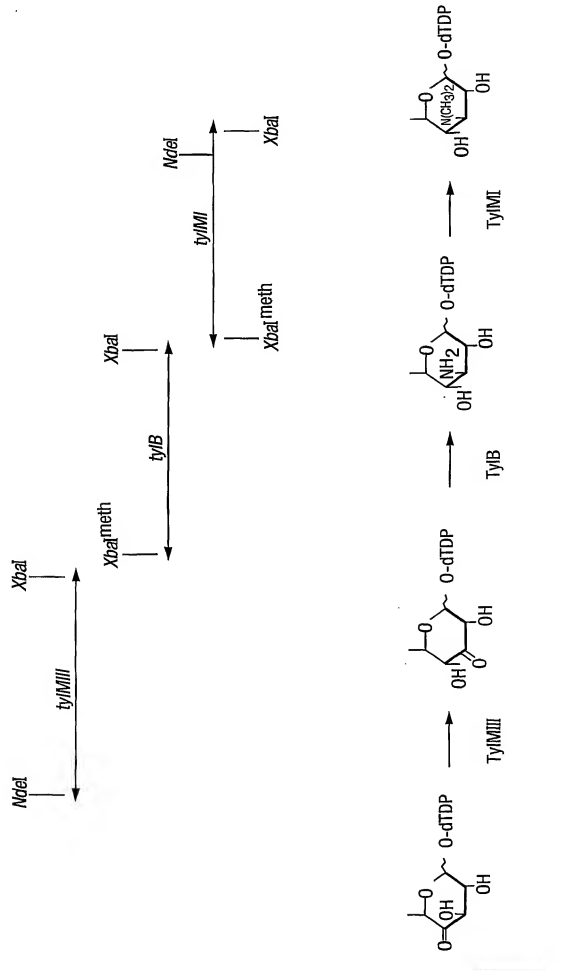


5-O-mycaminosyl-(4'-O-mycarosyl-)erythromycin A

```
  1 MTGLPRPAVRVPFHDLRDVHAATGVESEIGGALLRVAARGRYLLGAELAA 50
    |||||||||||||||||||||||||||||||| ||||||| ||||||||||
  1 MTGLPRPAVRVPFHDLRDVHAATGVESEIGAALLRVAAGGRYLLGAELAA 50

 51 FEERFAEYCGNAHCVAVGSGLDDARLALWALGVGEGDEVIVPSHTFIASW 100
    |||||||||||||||||||||| ||||||||||||||||||||||||||||
 51 FEERFAEYCGNAHCVAVGSGLDALRLALWALGVGEGDEVIVPSHTFIASW 100

101 LAVSATGATPVPVEPGDPGEPGPGAFLLDPDRLEAALTPRTRAVMPVHLY 150
    |||||||||||||||||||:|||||||||||||||||||||||||||||||
101 LAVSATGATPVPVEPGDPGQPGPGAFLLDPDRLEAALTPRTRAVMPVHLY 150

151 GHPVDLDPVGAFAEPHGLAVVEDAAQA.TARYRGRRIGSGHRTAFSFYPG 199
    |||||||||||||| |||||||||| |||||||||| ||||||| |||||
151 GHPVDLDPVGAFAERHGLAVVEDAAQAHGARYRGRRIGSGHATAFSFYPG 200

200 KNLGALGDGGAVVTSDPELADRLRLLRNYGAREKYRHEERGTNSRLDELQ 249
    |||||||||||||||||||||||||||||||||||||||||||||||||||
201 KNLGALGDGGAVVTSDPELADRLRLLRNYGAREKYRHEERGTNSRLDELQ 250

250 AAVLSVKLPYLDAWNTRRREIAARYGEALAGLPGVTVPEGRV.AEPVWHQ 298
    |||||||||||||||||||||||||||||||||||||||||| ||||||| |
251 AAVLSVKLPYLDAWNTRRREIAARYGEALAGLPGVTVPEAAAWAEPVWHQ 300

299 YVLRSPYRDRLRRRLAEAGVETLVHYPVAVHASGAYAGAGPCPAGGLPRA 348
    |||||||||||||||||||||||||||||||||||||||||||||||||||
301 YVLRSPYRDRLRRRLAEAGVETLVHYPVAVHASGAYAGAGPCPAGGLPRA 350

349 ERLAGEVLSLPIGPHLPDEAVEVVIAAVQSAALDSWEEGP 388
    |||||||||||||||||||||||||||||||||||||||
351 ERLAGEVLSLPIGPHLPDEAVEVVIAAVQSAALDSWEEGP 390
```

Fig. 33

Fig.34.


NdeI
tylMIII
XbaI
XbaImeth
tylB
XbaI
XbaImeth
tylMII
NdeI
XbaI
O-dTDP
TylMIII
TylB
TylMII

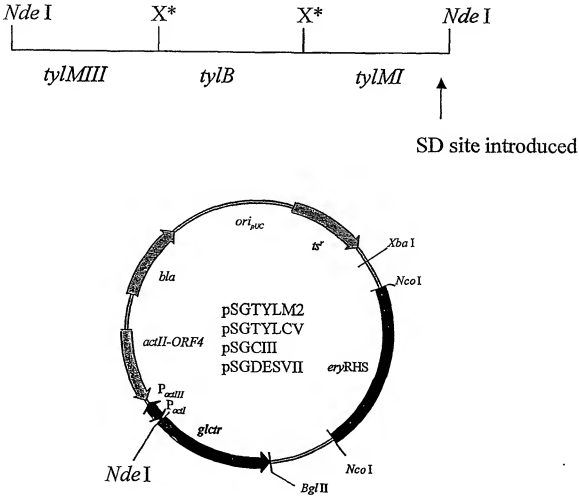
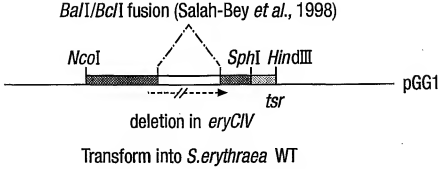

Nde I
X*
X*
Nde I
tylMIII
tylB
tylMI
SD site introduced
ori pUC
ts r
Xba I
Nco I
bla
pSGTYLM2
pSGTYLCV
pSGCIII
pSGDESVII
actII-ORF4
eryRHS
P actIII
P actI
glctr
Nde I
Nco I
Bgl II

Fig.35

Fig.36.



Two thiostrepton sensitive clones-no2 and 5-were isolated

1: PCR using WT genomic as template
2: PCR using no2 genomic as template
3: PCR using no3 genomic as template

← The *eryCIV* deletion is inserted into the genome

Σ: *S.erythraea* GG1 was isolated

Fig.37.

RT:0.00-24.99
SGQ2
mycEB
NL:
1.17E9
TIC F:+cESI
Full ms [
150.00-
1800.00] MS
SGQ2+mycEB
20.37
24.83
24.46
24.02
15.54
19.25
18.05
9.38
14.85
13.08
8.54
8.63
3.11
5.92
2.73
2.09
SGQ2pSGCIII
NL:
1.07E9
TIC F:+cESI
Full ms [
150.00-
1800.00] MS
deltaerycIV+
mycEB
23.72
21.32
24.49
20.31
m/z
750
17.80
14.82
12.83
13.09
7.88
8.65
3.83
2.74
1.05
Time (min)
deltaerycIV+mycEB#720 RT:21.29 AV:1 NL: 2.72E8
F:+c ESIFull ms [150.00-1800.00]
1403.1
3,5-di-O-mycarosyl-EB
m/z 713
713.3
511.0
672.7
818.1
983.3
1398.2
1418.6
1719.0
367.0
529.0
729.1
1259.2
1421.0
Relative Abundance
m/z

PCT/GB 01/01743

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P19/00 C12P19/62 C07H17/08 C12N1/00 C12N1/21
C12N15/54 C12N15/76

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C07H C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data, STRAND, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 23630 A (ABBOTT LABORATORIES) 3 July 1997 (1997-07-03) claims 1-22; figure 1B ---	1-59
X	M. DOUMITH ET AL.: "Interspecies complementation in Saccharopolyspora erythraea." MOL. MICROBIOL., vol. 34, no. 5, 1999, pages 1039-1048, XP001021052 cited in the application the whole document --- -/--	1-59

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 September 2001	12/09/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Douschan, K

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. GAISSER ET AL.: "A defined system for hybrid macrolide biosynthesis in Saccharopolyspora erythraea." MOL. MICROBIOL., vol. 36, no. 2, 2000, pages 391-401, XP001021072 the whole document	1-59
X	WO 96 40968 A (THE LELAND STANFORD JUNIOR UNIVERSITY) 19 December 1996 (1996-12-19) claims 1-36	1-59
X	US 3 697 547 A (ABBOTT LAB.) 10 October 1972 (1972-10-10) example 5	24,25,28
X	US 4 439 426 A (PIERREL S.P.A.) 27 March 1984 (1984-03-27) column 1; claim 8	24,25,28
X	EP 0 098 732 A (ELI LILLY AND COMPANY) 18 January 1984 (1984-01-18) claims 1-4	24,27
X	WO 91 16334 A (ABBOTT LAB.) 31 October 1991 (1991-10-31) claims 1-12	24,25
X	WO 98 49315 A (KOSAN BIOSCIENCES, INC.) 5 November 1998 (1998-11-05) figures 11,12A,12B	24-27
X	K. MILLER ET AL.: "Purification, cloning and heterologous expression of a catalytically efficient flavonol 3-O-galactosyltransferase." J. BIOL. CHEM., vol. 274, no. 48, 1999, pages 34011-34019, XP001021051 the whole document	29-59
X	GB 2 256 197 A (CIBA GEIGY AG) 2 December 1992 (1992-12-02) claims 1-19	29-59
X	WO 93 10248 A (NOVO-NORDISK AS) 27 May 1993 (1993-05-27) claims 1-15	29-59

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9723630 A	03-07-1997	US 5998194 A	07-12-1999
		EP 0874548 A	04-11-1998
		JP 2000502899 T	14-03-2000
WO 9640968 A	19-12-1996	US 5712146 A	27-01-1998
		AU 703920 B	01-04-1999
		AU 6157596 A	30-12-1996
		CA 2224104 A	19-12-1996
		EP 0871760 A	21-10-1998
		NZ 310729 A	29-09-1999
		US 6077696 A	20-06-2000
		US 6215007 B	10-04-2001
		US 5962290 A	05-10-1999
US 3697547 A	10-10-1972	NONE	
US 4439426 A	27-03-1984	IT 1139874 B	24-09-1986
		IT 1139875 B	24-09-1986
		AT 17351 T	15-01-1986
		BE 891734 A	30-04-1982
		CA 1169375 A	19-06-1984
		CA 1189856 A	02-07-1985
		CH 658250 A	31-10-1986
		DE 3268339 D	20-02-1986
		EP 0056291 A	21-07-1982
		FI 820057 A,B,	10-07-1982
		FI 834570 A,B,	13-12-1983
		FR 2502155 A	24-09-1982
		JP 1602510 C	26-03-1991
		JP 2024279 B	29-05-1990
		JP 57140779 A	31-08-1982
		US 4540662 A	10-09-1985
EP 0098732 A	18-01-1984	US 4423148 A	27-12-1983
		AT 18233 T	15-03-1986
		CA 1211731 A	23-09-1986
		DE 3362287 D	03-04-1986
		DK 307083 A	03-01-1984
		GB 2122993 A,B	25-01-1984
		GR 78600 A	27-09-1984
		IL 69093 A	30-09-1986
		JP 59027899 A	14-02-1984
		KR 8601997 B	12-11-1986
		US 4528369 A	09-07-1985
WO 9116334 A	31-10-1991	US 5141926 A	25-08-1992
		CA 2080583 A	19-10-1991
		EP 0525083 A	03-02-1993
		IE 911188 A	23-10-1991
		JP 2587562 B	05-03-1997
		JP 5504890 T	29-07-1993
		KR 9608668 B	28-06-1996
		PT 97390 A	31-01-1992
WO 9849315 A	05-11-1998	AU 732909 B	03-05-2001
		AU 7172298 A	24-11-1998
		EP 0979286 A	16-02-2000
		US 6117659 A	12-09-2000

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2256197 A	02-12-1992	AT 401940 B	27-12-1996
		AT 112792 A	15-05-1996
		AU 655470 B	22-12-1994
		AU 1705292 A	03-12-1992
		BE 1005579 A	09-11-1993
		CA 2070057 A	01-12-1992
		CH 685057 A	15-03-1995
		DE 4217616 A	03-12-1992
		DK 71992 A	01-12-1992
		ES 2046118 A	16-01-1994
		FI 922515 A	01-12-1992
		FR 2677040 A	04-12-1992
		GR 92100250 A	31-03-1993
		HU 65728 A,B	28-07-1994
		IE 921769 A	02-12-1992
		IT 1255044 B	17-10-1995
		JP 5199871 A	10-08-1993
		LU 88123 A	06-12-1993
		MX 9202583 A	01-11-1992
		NL 9200943 A	16-12-1992
		NO 922134 A	01-12-1992
		NZ 242958 A	25-06-1993
		PT 100545 A	31-08-1993
		SE 9201544 A	01-12-1992
		ZA 9203941 A	28-04-1993
WO 9310248 A	27-05-1993	EP 0672154 A	20-09-1995
		FI 942227 A	13-05-1994
		JP 7503363 T	13-04-1995